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Enzymatically-Mediated Co-Production of Cellulose Nanocrystals and Fermentable Sugars

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Abstract: Cellulose nanocrystals (CNCs) can be extracted from cellulosic materials through the degradation of non-crystalline cellulose domains in the feedstock via acid hydrolysis. However, the sugars released from the hydrolysis process cannot be easily recovered from the acid waste stream. In this study, cellulases were used to preferentially degrade non-crystalline domains with the objectives of recovering sugars and generating a feedstock with concentrated CNC precursors for a more efficient acid hydrolysis process. Filter paper and wood pulp substrates were enzyme-treated for 2–10 h to recover 20–40 wt % glucose. Substantial xylose yield (6–12 wt %) was generated from wood pulp. CNC yields from acid hydrolysis of cellulases-treated filter paper, and wood pulp improved by 8–18% and 58–86%, respectively, when compared with the original substrate. It was thought that CNC precursors accumulated in the cellulases-treated feedstock due to enzymatic digestion of the more accessible non-crystalline celluloses. Therefore, acid hydrolysis from enzyme-treated feedstock will require proportionally less water and reagents resulting in increased efficiency and productivity in downstream processes. This study demonstrates that an enzymatically-mediated process allows recovery of fermentable sugars and improves acid hydrolysis efficiency for CNC production.

Keywords: acid hydrolysis; cellulase; cellulose nanocrystals; fermentable sugars

1. Introduction

In recent years, the demand for print media products has been declining, especially in North America [1], due to the ever growing use and popularity of electronic media. Accordingly, the pulp and paper industry is aiming to diversify to high value-added products such as cellulose nanocrystals (CNCs). Cellulose, which makes up the major structural component of wood, is composed of poorly ordered amorphous chains, tightly packed crystalline regions and relatively less ordered para-crystalline domains [2]. Highly crystalline cellulose extracted in nanoscale dimensions of 3–5 nm width and 50–500 nm length are referred to as CNCs [3]. These cellulose crystals have been isolated from different sources such as wood pulp [4], cotton (Whatman™ No. 1 filter paper,) [5], tunicate (an aquatic invertebrate) [6], and bacteria [7]. CNCs are highly reactive and show a superior mechanical strength due to their large surface area as nanoparticles and high crystallinity, respectively [8]. The tensile strength (7.5 GPa) and axial elastic modulus (150 GPa) of CNCs are greater than or comparable with steel and Kevlar at low density (1.6 g/cm^3) [3,9]. Therefore, CNCs have potential application as biodegradable and renewable reinforcement fillers for composite

materials. CNC reinforced nanocomposites of cement [10], polylactic acid films [11], polyurethane foams [12], and adhesives [13] have shown improved mechanical properties. Cytotoxicity studies on different cell lines [14,15] show that CNCs are safe and biocompatible for biomedical applications such as diagnostic imaging [16] and drug delivery [17]. In addition, CNCs form a chiral nematic order that can be manipulated to form iridescent films with a potential application in developing colored films and sensors [18,19].

CNCs are isolated from a cellulosic feedstock by hydrolysis of amorphous celluloses with concentrated acid [3,20,21]. In this process, the acid protonates either the glycosidic or cyclic oxygen to promote nucleophilic attack of glycosidic bonds (and therefore cellulose chains) by water [22]. Under controlled conditions, acid hydrolysis reaches a plateau over the passage of time to generate CNC fragments that represent the acid recalcitrant crystalline cellulose [23]. Acid recovery methods are being introduced into CNC production processes to ensure economic feasibility and to reduce waste [24]. Mechanical methods can also be used to degrade amorphous cellulose by applying shear force along the length of the chain [25,26]. Recently, subcritical water has been explored for CNC extraction with promising results in terms of CNC yield and characteristics [27].

Even though acid hydrolysis has been well studied for CNC extraction, the methodology has some limitations. The major challenges of this process are: (a) the loss of reusable sugars from hydrolysis of amorphous cellulose in the acid waste stream, and (b) reaction of the acid with sugars in the waste stream reduces acid recovery efficiency [24]. These issues arise because of the high composition of amorphous cellulose in the feedstock, which has no contribution to CNC production. However, the recovery and reuse of sugars could improve the economics of the CNC production process. These limitations can be potentially addressed by introducing a cellulase enzymatic treatment to degrade the amorphous region before the cellulose is acid-hydrolyzed.

Cellulases are a set of enzymes that work in synergy to catalyze the degradation of celluloses. Hydrolytic cellulases are divided into three major groups: (a) endoglucanases break the amorphous cellulose chains to create chain ends; (b) exoglucanases/cellobiohydrolases bind to reducing/non-reducing ends of chains and release cellobiose units; and, (c) β-glucosidases/cellobiases breakdown cellobioses to glucose [28]. In addition, non-hydrolytic proteins, such as carbohydrate binding modules (bound to the enzymes) and free proteins, such as swollenin, expansins, and expansin-like proteins loosen tightly packed domains to facilitate enzyme accessibility [29].

Various reports show that cellulases rapidly hydrolyze amorphous cellulose [30–32]. Crystalline cellulose degradation is relatively delayed because cellulases are too large to enter the tightly packed core until the enzymes gradually loosen the structure [33]. There is potential to manipulate cellulase treatment to preferentially hydrolyze amorphous cellulose, which would facilitate the recovery of sugars and generate a feedstock with less amorphous fractions for a CNC extraction process. During cellulose degradation, the macro-structural organization of cellulose chains could likely limit the surface accessibility of the total amorphous domains. Detailed analysis of the cellulose structure shows that a fiber cell wall layer (1.7–3.7 μm) is made up of stacks of macrofibrils (500 nm) [34]. The latter is an aggregate of microfibrils (25 nm), which are the basic repeating units of the cellulose structure. Microfibrils are formed from bundles of elementary fibrils composed of tightly packed 36 parallel cellulose chains [35], with a decreasing level of crystallinity order from the core to the outer layer [36]. The elementary fibril is also interrupted with amorphous regions (dislocation sites) along the length [33]. This structural organization implies that amorphous and crystalline chains are interspersed in the fiber cell wall. During enzymatic digestion, the total amorphous celluloses are not simultaneously accessible as the cell wall is only gradually peeled off layer by layer [33,37]. Zhao et al. observed no change in the crystallinity of cotton cellulose, even after controlled acid hydrolysis to degrade amorphous cellulose, which suggested that amorphous cellulose in the core was not accessible [38].

Some studies have explored cellulase treatments to produce microfibrillated cellulose (MFC) via mechanical disintegration. MFCs are particles that have a nano-sized width, with lengths that could reach millimeters and interrupted by some less ordered celluloses [3]. Hydrolysis of less ordered cellulose with endoglucanase has improved the efficiency of mechanical homogenization

[39,40]. CNCs have been extracted from acid hydrolysis of MFCs, generated by the homogenization of sisal fiber that is treated with a mixture of all three groups of hydrolytic cellulases [41], from here on referred to as a cellulase cocktail. Zhu et al. reported that the crystallinity of cellulase cocktail-treated Kraft pulp improved with longer enzymatic treatment and CNC-like particles were generated by homogenization [42]. There were also efforts to produce CNCs solely by endoglucanase treatments [43,44]. Multiple cycles or high enzyme loadings are required to achieve significant CNC yield.

Most of these enzymatic hydrolysis studies mainly focus on the generation of MFC by mechanical disintegration, which requires multiple energy intensive passes, while acid hydrolysis is the most common CNC extraction process [3,20,21]. In addition, the prospect of recovering fermentable sugars could not be explored with monocomponent endoglucanases treatment, as these enzymes mostly act on amorphous cellulose, with limited saccharification [34]. To the best of our knowledge, a report by Beltramino et al. is the only account of a cellulase cocktail treatment study for CNC extraction via acid hydrolysis [45]. This study showed that enzymatic treatment increased the crystallinity of cotton linter due to the degradation of amorphous cellulose. This seems to indicate that better accessibility of the crystalline cellulose to the acid improved the CNC yield. The influence of the extent of enzymatic treatment on CNC yield and the prospect of co-generation of fermentable sugars are yet to be explored. Further investigation on this enzymatically-mediated process can give insights to the forestry and CNC industry to fully exploit this approach.

The aim of this study is to assess the effect of cellulase cocktail degradation of model substrate (Whatman™ No. 1 filter paper) and wood pulp feedstocks over a period of time on the recovery of fermentable sugars and acid hydrolysis efficiency. We hypothesize that the preferential degradation of amorphous cellulose can be achieved by manipulating hydrolysis time. Hence, the CNC precursor will be concentrated in the enzyme-treated cellulosic solid, which will improve the CNC yield from acid hydrolysis relative to the original material.

2. Results and Discussion

2.1. Cellulase Cocktail Dosages Response Curve

A glucose yield response curve as a function of cellulase cocktail loadings from 24 h hydrolysis was generated. The enzyme loading at the point when glucose yield started to plateau, with no further significant increase in yield at subsequent loadings, was identified as the most effective cellulase cocktail dosage. At this point, all of the enzyme-accessible binding sites would have been saturated [46]. Further addition of enzyme protein would not contribute to cellulose hydrolysis and only incur unnecessary cost to the process. Therefore, this effective cellulase loading was selected for subsequent enzymatic treatment investigation.

The effective cellulase dosage for the filter paper substrate was 1.7 fold higher when compared with wood pulp. Whatman™ No. 1 filter paper, which is made from cotton pulp [47], has more crystalline cellulose (83% crystallinity index, CI) fraction than hard wood pulp (73% CI) [48]. It has been previously suggested that the hydrolysis of crystalline cellulose requires higher loading for synergistic action of cellulases catalyzed by certain classes of exoglucanases [34].

2.2. Substrate Degradation Profile

Filter paper and wood pulp were treated with the cellulase cocktail (using the effective loading identified for the respective substrate) for 2–10 h. Fermentable sugars released in the enzyme hydrolysate and the undigested solids generated were assessed. Glucose yields from enzymatic treatment of filter paper ranged from 23.7 ± 0.2 to 42.0 ± 2.2 wt % substrate conversion (Figure 1a). After 6 h of treatment, there was no significant difference in yield ($p < 0.05$). A reduction in hydrolysis rate over time has been well reported in literature [49,50]. Based on the general understanding of cellulase enzyme kinetics, easily accessible amorphous cellulose is expected to be degraded rapidly during initial stages but hydrolysis rates level off when the residual cellulose becomes recalcitrant [34]. Xylose yield from filter paper was relatively poor and did not exceed 1.3%

(Figure 1a). Cotton is almost entirely composed of cellulose (98%) with only a small fraction of hemicelluloses (0.5%) [51].

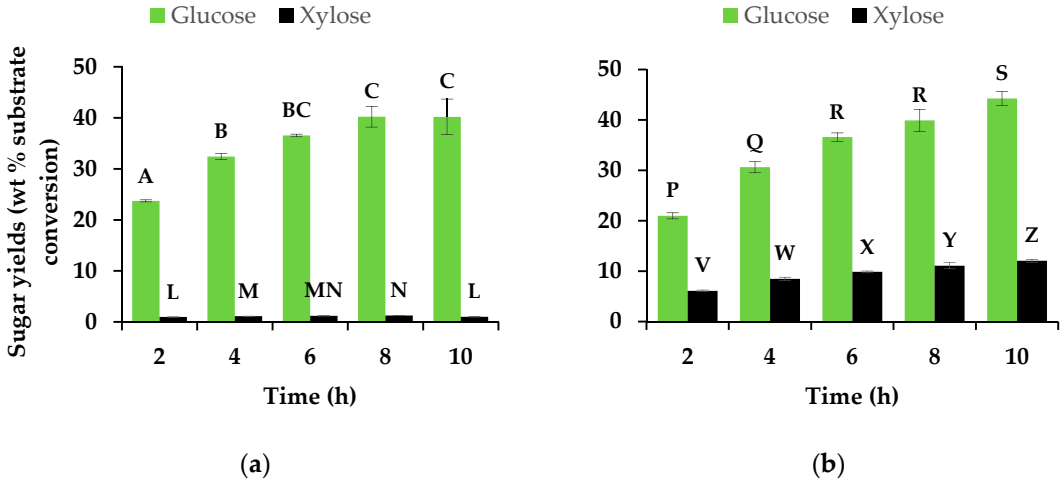

Figure 1. Glucose and xylose yields from cellulase cocktail treatment of (**a**) filter paper and (**b**) wood pulp over a time period of 2–10 h. Bars that are denoted by non-identical letters within each yield data are significantly different ($p < 0.05$). The cellulase loading for filter paper treatment was calibrated to 1.7 fold higher relative to wood pulp to ensure that enzyme concentration is not a limiting factor based on dosage response curve analysis.

Wood pulp hydrolysis released glucose (21.0 ± 0.6 to 44.2 ± 1.4 wt % substrate conversion) and xylose (6.1 ± 0.2 to 12.1 ± 0.3 wt % substrate conversion) during the examined period (Figure 1b). There was significant increase in the yields for both of the sugars over the 10 h treatment, except for glucose yield at 6 and 8 h ($p < 0.05$). This implies that there was no rate-limiting factor for enzyme hydrolysis at these time points. Xylose was the major five carbon sugar recovered because the wood pulp was sourced from hardwood feedstock [52]. Cellobiose was not detected from enzymatic hydrolysis of both substrates, but an unidentified oligosaccharide (with a retention time close to cellobiose in the HPLC chromatogram) made up less than 2 wt % conversion. This implies that the cellulase cocktail used in this study has sufficient β-glucosidase composition to hydrolyze cellobiose and prevents feedback inhibition for complete saccharification of cellulose [53].

The residual solids after enzymatic treatment were washed and gravimetric analysis of the freeze dried solid was carried out to calculate undigested solid (wt % substrate) to assess the mass loss (Table 1).

Table 1. Undigested solid and cellulose nanocrystal (CNC) yields from cellulase cocktail treated filter paper and wood pulp.

Enzymatic Treatment (h)	Filter Paper			Wood Pulp		
	Undigested Solid (wt % Substrate)	CNC Yield (wt % Acid-Hydrolyzed Feedstock)	Overall CNC Yield (wt % Original Feedstock)	Undigested Solid (wt % Substrate)	CNC Yield (wt % Acid-Hydrolyzed Feedstock)	Overall CNC Yield (wt % Original Feedstock)
0	99.5 ± 0.5 ^{A}	59.3 ± 0.1 ^{A}	59.0 ± 0.4 ^{A}	100.5 ± 0.5 ^{a}	10.3 ± 0.1 ^{a}	10.3 ± 0.1 ^{ab}
2	79.2 ± 0.2 ^{B}	63.8 ± 2.7 ^{B}	50.5 ± 2.2 ^{B}	74.5 ± 0.7 ^{b}	16.2 ± 1.5 ^{b}	12.1 ± 1.0 ^{a}
4	70.4 ± 0.4 ^{C}	65.2 ± 0.8 ^{B}	45.9 ± 0.6 ^{C}	62.7 ± 1.3 ^{c}	17.7 ± 0.9 ^{b}	11.1 ± 0.7 ^{a}
6	65.4 ± 0.2 ^{D}	66.7 ± 1.6 ^{BC}	43.6 ± 1.1 ^{C}	54.0 ± 1.4 ^{d}	19.2 ± 1.5 ^{b}	10.3 ± 0.7 ^{ab}
8	62.5 ± 2.3 ^{E}	67.2 ± 0.6 ^{BC}	42.0 ± 2.0 ^{C}	50.7 ± 0.2 ^{e}	17.3 ± 1.9 ^{b}	8.8 ± 1.0 ^{bc}
10	60.0 ± 0.2 ^{E}	69.9 ± 1.8 ^{C,*}	42.0 ± 1.2 ^{C,*}	45.4 ± 1.8 ^{f}	18.4 ± 0.7 ^{b}	8.3 ± 0.2 ^{c}

Values within columns that are denoted by non-identical letters (in superscript) are significantly different ($p < 0.05$); * Means and standard deviations were calculated from duplicates.

2.3. CNC Yield (wt % Acid-Hydrolyzed Feedstock)

The undigested solid was acid-hydrolyzed at a constant solid (8 g) to acid (100 mL) ratio to isolate CNCs. The effect of the enzymatic treatment on the acid hydrolysis process was determined by calculating the CNC yield from the reaction flask as wt % of acid-hydrolyzed feedstock. The CNC yields from 0 to 10 h cellulase cocktail treated-filter paper and wood pulp ranged from 59.3 ± 0.1 to 69.9 ± 1.8 and 10.3 ± 0.1 to 18.4 ± 0.7 wt % acid-hydrolyzed feedstock, respectively (Table 1). The enzymatic treatment increased the yields by 8–18% for filter paper and 58–86% for wood pulp over the studied enzyme treatment periods, as compared with the untreated feedstock (Table 2). These improvements suggest that there was significant accumulation of CNC precursor in the residual solids due to the relative inaccessibility of the crystalline cellulose [33] and the rapid degradation of amorphous cellulose by the cellulases [30–32]. In the case of wood pulp, hydrolysis of hemicellulose, which is an amorphous structure that does not contribute to the CNC product, also promotes CNC precursor concentration. Therefore, it is likely that the acid more efficiently hydrolyzed the concentrated CNC precursors in the cellulase cocktail-treated feedstock, and thus generated more CNC (wt % acid-hydrolyzed feedstock) relative to the untreated pulp/filter paper. Consequently, fewer sugars and their degradation products enter into the acid waste stream, which eases the acid recovery process [24].

Table 2. Improvement in CNC yield (%) from acid hydrolysis of cellulase cocktail-treated filter paper and wood pulp relative to the untreated feedstock.

Enzymatic Treatment (h)	CNC Yield Improvement (%)	
	Filter Paper	Wood Pulp
2	8 ± 4	58 ± 16
4	10 ± 1	72 ± 10
6	13 ± 3	86 ± 16
8	13 ± 1	68 ± 19
10	18 ± 3 *	79 ± 8

* Mean and standard deviation was calculated from duplicates.

If the effect of the enzymatic treatment on the process input for acid hydrolysis to extract a given amount of CNC is examined, then the feedstock requirement will decrease proportional to the % CNC yield improvement (Table 2). Consequently, the volumes and masses of water, and reagents (H_2SO_4 and NaOH) needed for acid hydrolysis and neutralization will also be correspondingly reduced. Furthermore, an increase in concentration of CNC from the acid hydrolysis reactor will enhance the throughput in the downstream purification process. Hence, the number of operation cycles for processes including centrifugation, tangential flow filtration, and drying can possibly be reduced. Therefore, the improvement in yield from the acid hydrolysis reaction due to the enzymatic treatment may significantly reduce the production cost of the acid hydrolysis and substantially conserve energy and time in the subsequent CNC purification processes. The enzymatically-mediated process will require more filter paper and wood pulp substrates than the untreated material, to generate the feedstock for acid hydrolysis to extract a given amount of CNC. This is due to the mass loss from enzymatic degradation of the substrates to sugars (undigested solids wt % substrates, Table 1). In the case of wood pulp, it is possible to offset additional costs from the recovered sugar co-products, which currently has a slightly higher value ($838 USD/metric ton dextrose) [54] than the feedstock ($817 USD/metric ton) [55].

When the two feedstocks were compared, irrespective of the enzyme treatment, CNC yield from filter paper was significantly higher (up to 6 fold) than wood pulp. Previous studies on cotton feedstock have reported CNC yields ranging from 23 to 65% [45,51]. This implies that filter paper has a significantly higher composition of CNC precursors with high crystallinity than wood pulp [48], as discussed previously (Section 2.1).

During the course of the enzymatic treatment period, there was no significant improvement in CNC yield (wt % acid-hydrolyzed feedstock) over time from acid hydrolysis of cellulase cocktail-treated substrates (except the 10 h enzyme-treated filter paper, Table 1). This indicates that enzymatic treatment did not show exclusive preference towards amorphous cellulose. Even though

the total amorphous celluloses in the cell wall would have been relatively easy to hydrolyze [30–32], amorphous chains buried inside the fiber can only be gradually accessible as cellulases peel off layers of the cell wall [33,37]. Zhao et al. reported that these amorphous celluloses in the core of the fiber were inaccessible even to controlled acid hydrolysis [38]. Therefore, it is likely that CNC precursors exposed to enzymatic attack are simultaneously disintegrated by amorphogenesis inducing proteins [29] and hydrolytic cellulases (with or without complete degradation). On the other hand, the improvement in CNC yield relative to the untreated substrate suggest that the disintegration and/or dissolution rate of the CNC precursors, is relatively much slower due to recalcitrance [33]. During this delay, the peeling off action by unbound enzymes and amorphogenesis inducing proteins could possibly expose new amorphous cellulose layers that are simultaneously degraded at a faster rate. It is also possible that acid hydrolysis conditions were too aggressive for the cellulase cocktail-treated feedstock, leading to the dissolution of CNC precursors. The original substrate has layers of amorphous and para-crystalline cellulose enclosing the CNC crystalline core [2,36]. In cellulase-treated substrates, these layers were likely softened (amorphogenesis) and/or completely removed in a time dependent manner, with longer treatments exposing more CNC precursor for acid hydrolysis. Improved accessibility to acid could cause dissolution of cellulose chains, instead of fragmentation, along the length or the surface of the CNC precursor, and thus reduce for CNC yield. Therefore, milder acid hydrolysis conditions for a cellulase-treated feedstock should be investigated in the future.

2.4. Overall CNC Yield (wt % Original Feedstock)

The overall CNC yield is defined as the wt % of CNC that can be extracted from the starting material by accounting for the mass loss due to enzymatic saccharification of cellulose chains. This data gives an indication of CNC precursor disintegration or dissolution during enzymatic and/or acid hydrolysis relative to the untreated feedstock. The overall CNC yield from cellulase cocktail treated filter paper significantly decreased when compared with the untreated substrate and stabilized after 4 h (Table 1). The implication is that enzymatic treatment and/or acid hydrolysis conditions have led to significant degradation of CNC precursors in the filter paper substrates. This also supports our interpretation for the stable CNC yield (% acid-hydrolyzed feedstock) as a function of enzyme treatment period. However, in the case of cellulase cocktail-treated wood pulp, there was no significant difference in the overall CNC yield from 2 to 8 h relative to the untreated feedstock. This was apparent despite the loss of up to half the weight of the substrate at 8 h enzymatic hydrolysis (50.7 ± 0.2 wt % substrate of undigested solids recovered, Table 1). It can be suggested that there was a relatively selective amorphous celluloses degradation in wood pulp substrate. The 10 h time period was possibly the turning point when CNC precursors were equally accessible for disintegration and/dissolution by the enzymes and/or acid hydrolysis.

As discussed previously in Section 2.3, filter paper has significantly more CNC precursor than wood pulp. There is a very high probability for the CNC precursors to serve as substrates for enzymatic dissolution due to their abundance in filter paper than in wood pulp. This was apparent even during the very early stages of enzymatic hydrolysis (2 h) of filter paper, which consequently reduced the overall CNC yield by 17% relative to the undigested substrate. There was no significant change in overall CNC yield from 4 to 10 h enzymatic treatment, possibly due to the recalcitrance of the residual solid. The stabilization of enzymatic hydrolysis observed during later stages, as evidenced by the sugar (Figure 1a) and undigested solid yields (Table 1), supports this argument.

Beltramino et al. studied the effect of enzymatic treatment of cotton linter on the overall CNC yield [45]. Significant yield improvement was reported from hydrolysis of cellulase cocktail-treated cotton with 62 wt % H_2SO_4 for 45 min. It was suggested that selective degradation of amorphous cellulose by the enzymes improved the accessibility of the CNC precursors to acid hydrolysis. Interestingly, the enzymatic treatment did not significantly change the CNC yield when 64 wt % acid concentration was used. In the present study, acid resistant solids recovered after acid hydrolysis of cellulase cocktail-treated feedstock ranged from only 1–3 wt % acid-hydrolyzed feedstock (Table 3). This suggests that accessibility to CNC precursors is unlikely to be a limitation to

isolate CNCs at longer acid hydrolysis time (≥2 h) relative to the study by Beltramino et al. (45 min). Therefore, the enzymatic treatment is not expected to improve the overall CNC yield (wt % original feedstock) in the present study. Preferential hydrolysis of amorphous celluloses by the cellulase cocktail cannot increase the native CNC precursor composition in the original feedstock. The aim of the enzymatic treatment was to concentrate the CNC precursors. Improvement in acid hydrolysis was implied by the increase in CNC yields (wt % acid-hydrolyzed feedstock) from this cellulase cocktail-treated feedstock.

Table 3. Over-sized reject from acid hydrolysis of cellulase cocktail-treated filter paper and wood pulp.

Enzymatic Treatment (h)	Over-Sized Reject (wt % Acid-Hydrolyzed Feedstock)	
	Filter Paper	Wood Pulp
0	1.02 ± 0.02	2.62 ± 0.11
2	1.24 ± 0.02	ND
4	1.69 ± 0.02	ND
6	2.02 ± 0.04	1.70 ± 0.10
8	1.71 ± 0.05	1.98 ± 0.06
10	1.50 ± 0.00 *	1.41 ± 0.03

ND, not determined; * Means and standard deviations were calculated from duplicates.

CNC and fermentable sugars obtained from the enzymatically-mediated process were totaled to assess the % total value-added product yield from the original feedstock. Yields rose from 59.0 ± 0.4% up to 85.0 ± 3.3% for filter paper and from 10.3 ± 0.1% up to 64.6 ± 1.8% for wood pulp, relative to the untreated feedstock (Figure 2). There was no significant difference in yield between 6–10 h for filter paper substrate, while further enzymatic hydrolysis of wood pulp to sugars at 10 h significantly increased the yield ($p < 0.05$). Co-generation of sugars and enhanced acid hydrolysis efficiency can improve the process economics of the CNC industry. However, reduction in the overall CNC yield could likely affect the economic feasibility of using filter paper as feedstock. The market value of CNC can reach up to $1000 USD/kg (Blue Goose Biorefineries Inc., Saskatoon, SK, Canada) [56], which is substantially higher when compared with $0.84 USD/kg cost for dextrose [54]. In the case of wood pulp substrate, over the course of 2–8 h enzymatic treatment, the overall CNC yield was not compromised. These results are very promising and further techno-economic assessment can give insights on the potential of the enzymatically-mediated process for wood pulp feedstock.

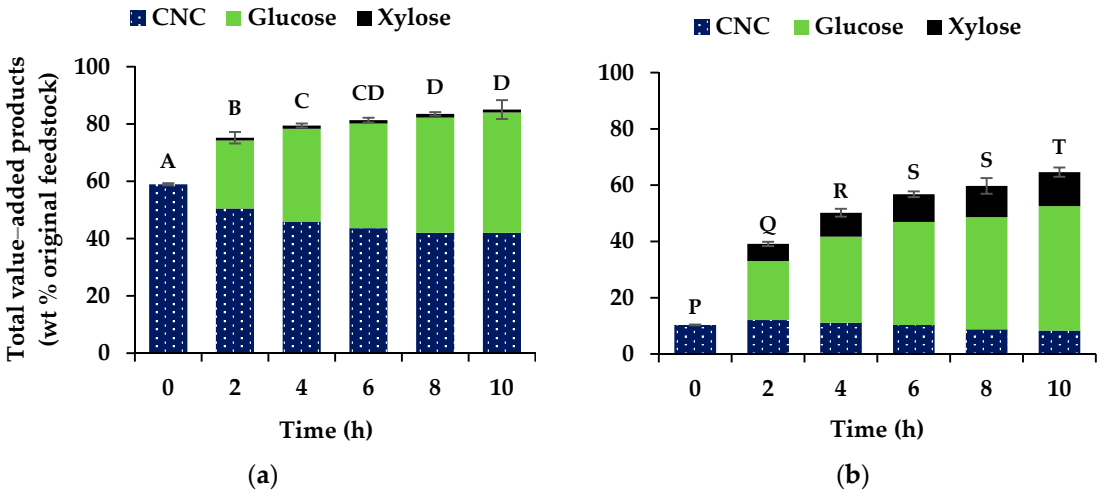

Figure 2. Total value-added products (CNC and sugars, wt % original feedstock) extracted from (**a**) filter paper and (**b**) wood pulp, via enzyme-mediated CNC production process. Error bars represent standard deviation of the total value-added products (wt % original feedstock). Bars that are denoted by non-identical letters are significantly different ($p < 0.05$). Means and standard

deviations for CNC yield from filter paper, cellulase cocktail treated for 10 h, were calculated from duplicates.

2.5. Structure of CNC under Transmission Electron Microscope

CNC particles isolated from the untreated and enzyme-treated feedstock exhibited nanoscale size in both length and width (Figure 3). The particles from both feedstocks had elongated needle-like shape. CNC isolated from wood pulp appeared to have narrower width as compared with CNC particles from filter paper.

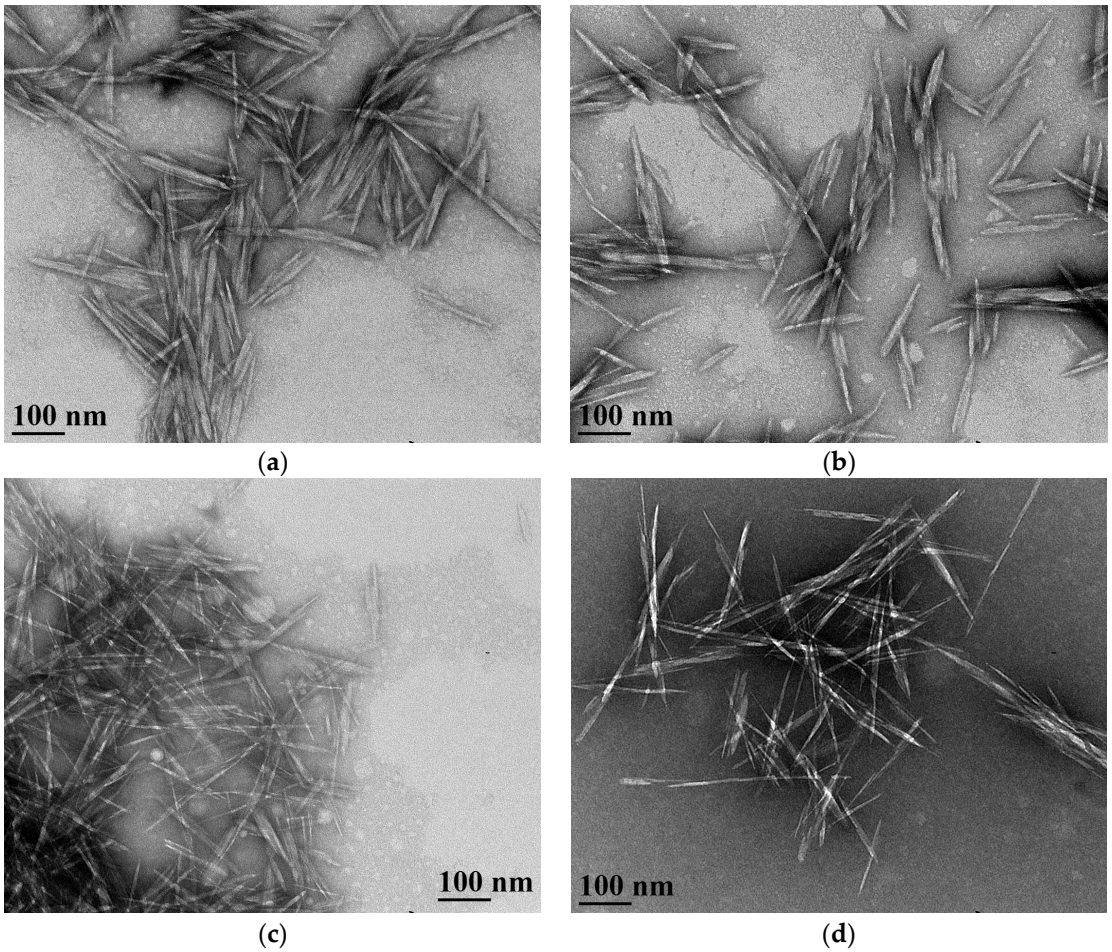

Figure 3. Transmission Electron Microscope (TEM) image of CNC isolated from: (**a**) untreated filter paper; (**b**) 8 h enzyme-treated filter paper; (**c**) untreated wood pulp and (**d**) 8 h enzyme-treated wood pulp.

3. Materials and Methods

Whatman™ No. 1 Qualitative filter paper (110 mm diameter, Whatman™ supplied by Fisher Scientific, ON, Canada) was used as model cellulose substrate. It was cut into approximately 4 mm × 27 mm strips with a paper shredder (Aurora AS 650 C, Torrance, CA, USA). Northern Bleached Hardwood Kraft (NBHK) pulp, predominantly composed of Aspen, was kindly provided by Alberta Pacific Forest Industries Inc. (Al-Pac, Edmonton, AB, Canada). The pulp was supplied as pressed and dried squares (approximately 6 mm × 8 mm) produced by a pulp chopper (Pierret Cutting Machine G45L1, Corbion, Belgium). This material was chosen as it is the same cellulose-based feedstock used for CNC production at the CNC pilot plant facility of our research collaborator, InnoTech Alberta (Edmonton, AB, Canada). The pulp was composed of 79.1 ± 1.0% cellulose, 21.2 ± 0.6% hemicellulose (xylan) and 4.0 ± 0.1% lignin, as determined by a two-step acid hydrolysis procedure [57]. It is likely that this lignin content (acid soluble lignin) from UV

absorption analysis was overestimated due to interferences from sugar degradation products [58]. A cellulase cocktail enzyme solution (NS 51129), a non-commercial proprietary research formulation with cellulosic biomass saccharification activity, was kindly provided by Novozymes® A/S (Bagsvaerd, Denmark). H_2SO_4 (95–98%) and NaOH (98.8%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific, respectively. Transmission Electron Microscope (TEM) imaging was carried out at the Advanced Microscopy Facility (AMF) in the Department of Biological Sciences, University of Alberta (Edmonton, AB, Canada).

3.1. Enzymatic Hydrolysis

3.1.1. Cellulase Cocktail Load

Filter paper assay was conducted to determine the activity (FPU/mL) of the enzyme solution [59]. Substrates were hydrolyzed at different cellulase cocktail loadings (FPU/g of substrate) for a constant time period to generate a dosage response curve. This study was used to identify a cellulase cocktail loading with the most effective activity, which was defined as the least loading with the maximum increase in glucose yield. Each substrate (10% solid consistency, *w*/*v*) was hydrolyzed with five enzyme loadings buffered in 0.05 M sodium citrate solution at pH 4.8 in 250 mL shake flask (5 g in 50 mL). The flask was incubated for 24 h in a shaking water bath at 50 °C, 100 rpm. The enzyme activity was terminated by heating in boiling water for 15 min.

3.1.2. Enzymatic Treatment

Batch enzymatic hydrolysis of filter paper and wood pulp were studied over a period of 2–10 h. Enzymatic hydrolysis was carried out under the same conditions as described in Section 3.1.1 (but scaled up to 500 mL shake flask, 20 g in 200 mL), using a cellulase cocktail loading with the most effective activity for each substrate. Untreated substrates were suspended in buffer (without addition of enzymes) and were mock treated under the same conditions.

3.2. Undigested Solids and Sugar Analysis

The slurry produced after enzymatic treatment was centrifuged at 33,700× *g* for 15 min at 20 °C. The precipitated solid pellet was re-suspended in water, washed three times by centrifugation and freeze-dried. Undigested solid yield (wt % original feedstock) was calculated using Equation (1), in which *W*1 is the mass of freeze-dried solid recovered after enzymatic treatment (g) and *W*2 is the mass of substrate before enzyme hydrolysis (20 g). The liquid enzyme hydrolysate was analyzed for glucose and xylose sugar yields through High Performance Liquid Chromatography (HPLC, Agilent 1200, Santa Clara, CA, USA) coupled with a Refractive Index Detector (RID, Agilent 1100, Santa Clara, CA, USA). Sugars in the sample (30 µL injection volume) were separated on HPX-87P column (Bio-Rad Aminex, Hercules, CA, USA) with water as the mobile phase at a flow rate of 0.5 mL/min at 80 °C for 40 min. The sugar yield (wt % conversion) for each identified sugar was calculated using Equation (2), in which *W*3 is the mass of recovered sugar (g).

$$\text{Undigested solid} = \frac{W1}{W2} \times 100\%, \quad (1)$$

$$\text{Sugar yield} = \frac{W3}{W2} \times 100\%, \quad (2)$$

3.3. Acid Hydrolysis for CNC Isolation

A standard operating procedure developed by InnoTech Alberta for bench-scale acid hydrolysis and CNC purification was adopted. The cellulase cocktail-treated solid (8 g) was hydrolyzed with 64 wt % H_2SO_4 (100 mL) in 500 mL shake flasks (8% pulp-to-acid ratio, *w*/*v*). The reaction was carried out in a water bath at 45 °C for 2 h with overhead stirring at 200 rpm. The acid was diluted 10 fold (*v*/*v*) with cold water to terminate the reaction. The suspension was centrifuged at 6400× *g* for 10 min to reduce the dilute acid volume. The pellet was re-suspended in water (final volume 150–200 mL)

and neutralized with NaOH (30%, *w*/*v*) to pH 7 on an ice bath. The suspension was centrifuged at 3700× *g* for 10 min to remove salts in the liquid phase, formed by neutralization. The pellet was further washed by re-suspending in water followed by centrifugation. The pellet was again re-suspended in water (final volume 25–75 mL) and dialyzed against water in regenerated cellulose membrane tube (Spectrum™ Spectra/PorTM, Rancho Dominguez, CA, USA, 12–14 KD molecular weight cut off). Dialysis was monitored for 3–5 days, using a conductometer until the ionic strength of the liquid dropped to 100–150 μS/cm for CNC particles to suspend. The suspension was centrifuged at 8900× *g* for 10 min to precipitate over-sized particles. The CNCs in the supernatant were collected as colloid and the pellet was re-suspended in water and centrifuged again to extract any remnant CNC at lower ionic strength. The supernatants were pooled and an aliquot sample (by weight) was oven dried overnight at 103–105 °C. The precipitated pellets were also pooled together and freeze-dried to analyze the amount of over-sized reject cellulosic material that were not hydrolyzed by the acid to CNC.

Equations (3)–(5) were used to calculate (a) CNC yield (wt % acid-hydrolyzed feedstock) from the acid hydrolysis reaction of a fixed mass of feedstock; (b) over-sized rejects (wt % acid-hydrolyzed feedstock); and, (c) overall CNC yield (wt % original feedstock) accounting for the mass loss due to enzymatic treatment, respectively, in which *W*4 is the mass of oven dried CNC from an aliquot sample (g), F is the ratio of total mass of colloid to mass of aliquot, *W*5 is the mass of the feedstock added to the acid hydrolysis reaction (8 g), *W*6 is the freeze-dried mass of the un-hydrolyzed pellet collected after acid hydrolysis (g) and US is the undigested solid (wt % substrate). In addition, the total value-added products (wt % original feedstock) were calculated as the sum of the overall CNC yield (wt % original feedstock) and sugar yield (wt % substrate conversion).

$$\text{CNC yield} = \frac{W4 \times F}{W5} \times 100\%, \quad (3)$$

$$\text{Over sized reject} = \frac{W6}{W5} \times 100\%, \quad (4)$$

$$\text{Overall CNC yield} = \frac{\text{CNC yield} \times \text{US}}{100}. \quad (5)$$

3.4. Transmission Electron Microscope Imaging

Freeze dried CNC was re-suspended in water (0.1%, *w*/*v*) and a droplet was mounted on a 300 mesh copper grid with a formvar film (Ted Pella Inc., Redding, CA, USA). After 10 min, the droplet was blotted off with filter paper. The specimen was stained with phospho-tungstic acid (2%, *w*/*v*) for 15 s. The droplet was blotted off with filter paper and images were taken using Philips/FEI, Morgagni 268 TEM (Hillsboro, OR, USA), operating at 80 kV.

3.5. Statistical Analysis

All data in Tables and Figures were reported as mean ± standard deviation, calculated from experimental triplicates. The means were compared with one-way analysis of variance (ANOVA) in conjunction with Tukey's test ($p < 0.05$) on Minitab® 17 statistical software (Version 17.3.1, Minitab Inc., State College, PA, USA).

4. Conclusions

Cellulase cocktail treatment of filter paper and wood pulp over a course of time allowed for the recovery of a range of fermentable sugars that can offset CNC production costs. CNC was extracted from the residual solids generated from a varying extent of enzymatic hydrolysis as a function of time. The CNC yield from acid-hydrolyzed filter paper and wood pulp improved by 8–18% and 58–86%, respectively, from 2 to 10 h enzymatic treatment. These results suggest that enzymatic treatment enhanced the acid hydrolysis efficiency, which can reduce reagent and operation costs for the CNC industry. The overall CNC yield, accounting for mass loss due to enzymatic treatment, decreased for filter paper substrates, but there was no change in yield during 2–8 h treatment of

wood pulp, which implied the selective hydrolysis of amorphous celluloses. Nevertheless, total recovery of value-added products increased significantly through the enzymatic treatment. This study demonstrated the promise of using an enzymatically-mediated approach in improving acid hydrolysis efficiency for CNC extraction and co-generation of fermentable sugars.

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