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INVESTIGATION INTO THE STRUCTURAL AND THERMAL BEHAVIOR OF BACTERIAL CELLULOSE FIBERS AFTER BIOLOGICALLY RELEVANT PURIFICATION

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ABSTRACT

Cellulose is the most abundant biopolymer on the planet. Historically rooted in the paper industry, advancements in colloidal chemistry, polymer chemistry, and the development of novel saccharification techniques have expanded the commercial applications of cellulose to include the production of liquid crystal displays, use in high strength

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composites, and biofuels. Despite this renewed interest in cellulosic products, the establishment of cellulose as a global commodity is significantly hindered by the inefficiencies in cellulose liberation and processing. The current model associated with cellulose liberation from lignin and hemicellulose relies on the use of highly basic reagents resulting in significant alterations to cellulose native structure. Laboratory techniques have been developed to attempt to isolate cellulose, while leaving it in its native structure. In this work, we demonstrate how even mild laboratory isolation techniques significantly influence cellulose structure in bacterial cellulose. Furthermore, we propose that bacteria cellulose serves as a model for cellulose as found in plants and animals.

Keywords: *Acetobacter xylinum, Gluconacetobacter hansenii* 23769, bacterial cellulose, crystallinity and alkalization, cellulose I, cellulose II, purification.

INTRODUCTION

Cellulose is the most abundant renewable natural resource on the planet [1]. Understanding the fundamental structure and chemistry of cellulose and cellulose ultrastructure is important for understanding end use applications of cellulose. For example, cellulose structure has impact on the ultimate strength of materials made from cellulose, on the dimensional stability of fibers and paper, and on the ability of enzymes to degrade cellulose into sugars as precursors for fuels and other chemicals [2,3]. The isolation and characterization of cellulose in its most native state, is the first step in deconstructing the complex biochemical interactions that occur between hemicellulose, lignin and cellulose *in vivo*. A better understanding of *in vivo* lignocellulosic chemistry would aid in the development of more efficient liberation techniques. This study aims to establish a novel bacterial cellulose (BC) purification scheme that preserves the inherent properties of *in vivo* cellulose (native cellulose), with the intent of developing a model for lignocellulosic chemistry.

Glucanobacter hansenii (*G. hansenii*) synthesizes highly crystalline cellulose that is chemically identical to plant based cellulose [3,4]. Under static conditions, *G. hansenii* produces the highest yields of cellulose in the form of a cellulose pad referred to as a pellicle [5]. Similar to plant based cellulose, BC pellicles must undergo purification. BC purification removes secondary organic material such as bacterial cells, proteins, media components and other soluble polysaccharides [6]. The most common method of purification is the exposure of the pellicle

to sodium hydroxide (alkali) solutions that range in concentration from 1% to 17% (w/w). The immersion of the pellicle in sodium hydroxide effectively dissolves the secondary organics associated within the pellicles [7]. However, the structure of native cellulose may be significantly altered.

During the process of mercerization, the conformation of the cellulose is irreversibly changed from cellulose I polymorph to cellulose II polymorph [3]. For some industrial applications, the transformation of cellulose I to the more thermal stable cellulose II is desired. However for the isolation of native cellulose, this is an undesired side effect.

Proteinase K (PK), named for its ability to digest keratin, is a highly active protein digesting enzyme (protease) that was first isolated in 1974 from fermenting bovine horn chips in the presences of manure. The isolation of the protease from the fungus *Tritirachium album Limber* was achieved by submerging the fungus in rich media under fermentative conditions and collecting secreted proteins. PK was isolated for its high proteolytic activity against keratin and its ability to digest native proteins discriminately by the hydrolysis of peptide bonds under physiologically relevant conditions. The complete biochemical characterization of PK led to the development of a commercially available protease that revolutionized biochemical assays aimed at the preservation of cellular components [8]. More specifically, when mixed with cellular extract, PK works to digest protein without disturbing cellular components associated with a high degree of hydrogen bonds (Nucleic Acids) [9]. The utilization of PK for the purification of bacterial cellulose may provide a novel technique for the isolation of cellulose in its most native state. As detailed in the literature, the structural characterization of cellulose requires the processing of the raw cellulose in biologically relevant manner that does not disrupt native hydrogen bonding. PK exhibits a high degree of proteolytic activity against native proteins without the addition of chemicals or heat treatment. Based on the literature, this technique should minimize alterations of the cellulose structure due to the purification method.

In this work, we present a novel technique for removing organic impurities from BC using the protein degrading enzyme PK. This method works to preserve cellulose in its most native conformation with future aspirations of modeling the unique biochemical characteristics of the *in vivo* associated cellulose I polymorph. We also demonstrate the inefficiencies associated with the current model of cellulose liberation. Structural comparisons performed on PK treated BC and alkali treated BC using thermal gravimetic analysis (TGA), differential scanning calorimetry (DSC) and X-ray diffraction (XRD) illustrate how the current model of cellulose liberation results in the enhanced resistance of the cellulose to enzyme mediated saccharification. We also examine the effects on the fibril structure resulting from the various isolation methods.

MATERIALS AND METHODS

Bacterial strain and growth conditions

The strain of *Gluconacetobacter hansenii* that was used in this study was ATCC 23769 obtained from the American Type Culture Collection, Manassas, VA (ATCC). The strain was cultured and plated in accordance with the provided ATCC protocol. For this study a primary 250 ml culture was established using Hestrin and Schramm media (SH) [10] under static conditions and maintained at 30ºC. To produce pellicles of equal size and depth, disposable petri dishes containing 30 ml of SH media were inoculated with 1 ml of primary culture. The plates were incubated at 30ºC for 14 days. SH media components: 12% citric acid, 2% glucose, 0.5% peptone, 0.27% sodium phosphate (dibasic), 0.5% yeast extract and 0.57% magnesium sulfate.

BC purification

Pellicles were removed from petri dishes and purified by 1 M base treatment (BH-BC), base only treatment (BO-BC) or Proteinase K treatment (PK-BC) defined in Table 1. At the start of each method, the pellicles were rinsed thoroughly and soaked in diH_2O for 24 h to remove loose organic debris. For the proteinase K treatment method, the BC pellicle was rinsed and soaked for 24 h at 4ºC. The BH-BC pellicles were immersed in a boiling 1 M solution of NaOH for 2 h. BH-BC were allowed to cool to room temperature then rinsed in diH_2O and

Table 1. Bacterial cellulose purification schemes

Sample ID	*Overnight Pre-rinse conditions*	*1 M NaOH*	*Heat (Boiling) 2 (h)*	*Proteinase K*	*Post Rinse conditions*
BH-BC	diH_2O	+	+	–	diH_2O
BO-BC	diH_2O	+	–	–	diH_2O
PK-BC	diH_2O at 4ºC	–	–	+	diH_2O at 4ºC
PKB-BC	diH_2O at 4ºC	–	+	+	diH_2O at 4ºC
Tris-BC	diH_2O	–	–	–	diH_2O
Utr-BC	diH_2O	–	–	–	diH_2O
NP-BC	None	–	–	–	None

All purification schemes were performed prior to freeze drying.

placed in 255 mM acetic acid bath overnight. The following day acid washed BH-BC were rinsed in diH_2O until a pH of 7 was reached [11]. Pellicles treated by base only method BO-BC, were immersed in a 1 M solution of NaOH and placed at 37ºC overnight with gentle stirring. BO-BC were rinsed thoroughly and washed in 255 mM acetic acid for 24 h followed by a final rinse step to achieve a pH of 7 [12]. Pellicle purified by Proteinase k method (PK-BC), post 4ºC rinse, were placed in petri dishes with 30 ml of 50 mM tris-HCl buffer (pH 8) containing 100 μg μl^{-1} Proteinase K (New England Biolabs, Ipswich, MA). Petri dishes were gently stirred for 24 h at 37ºC. PK-BC were removed from petri dishes and rinsed thoroughly in sterile diH_2O at 4ºC with gentle stirring. Heat treated proteinase K purified BC pellicles (PKB-BC) were created by boiling PK treated BC before drying. Additionally, tris-HCl buffer treated BC pellicles (Tris-BC) and diH_2O rinsed BC pellicles (Utr-BC) were generated by removing pellicles from growth medium and rinsing overnight with a solution of 50 mM tris-HCl buffer or diH_2O respectively. For comparisons and as a control, an untreated set of BC (NP-BC) was also prepared were pellicles were simply removed from growth medium and dried. All samples were placed in 50 ml tubes or cryogenically homogenized, frozen by liquid nitrogen immersion and freeze dried. Freeze dried samples were placed in a desiccator and held under vacuum. The microcrystalline plant derived cellulose, Avicel PH-101 obtained from FMC Corp., (Philadelphia, PA), purified and regenerated using commercial purification techniques described previously, was used as a control [13].

Cryogenic homogenization

After initial processing, a set of BC samples were cryogenically homogenized using a sterilized, pre-frozen mortar and pestle. Pellicles were placed in center of pestle and immersed in liquid nitrogen. Homogenized BC was placed in sterile pre-frozen tube, cryogenically stored in liquid nitrogen and freeze dried.

Chemical analyses

Full and cryogenically homogenized 3 mg freeze dried samples isolated from separate pellicles were analyzed in triplicate with two independent experimental repeats (n = 6) by attenuated total reflection (ATR) and Fourier transform-infrared spectroscopy (FT-IR), respectively, using a Nexus FT-IR 670 in the range of 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1} and after accumulation of 32 and 64 scans, respectively. Cryogenically homogenized samples for FT-IR were pressed at 670 MPa for 10 min using a Perkin Elmer pellet press (Perkin Elmer, Waltham, MA) into 1.5 mm thick disk with a diameter of 13 mm. Elemental composition (C,N) of freeze dried BC, was determined by dry combustion

elemental analysis using a Leco True-Specs CHN elemental analyzer. As a reference sample, Avicel PH-101 microcrystalline cellulose (FMC BioPolymer Philadelphia, Pennsylvania, USA) was used for comparison to a widely available cellulose sample. The analysis was performed in triplicate with each sample set representing three independent pellicle isolates (n = 3).

Thermal analysis

Thermal gravimetric analysis (TGA) was performed with a TGA Q500 (TA Inc., New Castle, DE) on 20 mg freeze dried samples under air conditions. Each sample data point represents three separate pellicle isolates (n = 3) The temperature range was 30ºC–800ºC and with a heating rate of 10ºC min^{-1} with an isothermal hold at 105ºC and 800ºC for 10 min. Differential scanning calorimeter (DSC) was performed on 3 mg freeze dried samples using a DSCQ100 (TA Inc., New Castle, DE) with standard sealed aluminum pans (part no. 900786.901). The temperature range was 25ºC–315ºC or 25ºC–350ºC with a heating rate of 10ºC min^{-1} followed by an isothermal hold for 5 minutes at 315ºC or 350ºC. Samples analyzed in the 25ºC–315ºC range were subjected to a second temperature ramp using the same thermal protocol. An empty sealed pan was used as reference. Each sample data point represents three separate pellicle isolates. (n=3)

Structural analysis

X-ray diffraction (XRD) was performed with a Rigaku Smartlab XRD (Rigaku, The woodlands, TX) using CuKα radiation generated at 40 kv and 44 mA. The Bragg angle of 2θ was scanned from 9º to 41º with a step and exposure time of 5 s. Freeze dried 20 mg samples were used for analysis. Crystalline index value of treated BC was calculated from XRD spectra using amorphous region subtraction. Each sample data point represents three separate pellicle isolates (n=3). The resulting spectra were analyzed and normalized and averaged for comparison. Field emission scanning electron microscopy (FE-SEM) was performed on purified BC using a JEOL 6400 cold field emission microscope (JEOL, Peabody, MA, USA). Images were analyzed using ImagePro 4.5 software (MediaCybernetics, Rockville, MD). All images were converted to 8-bit gray level images through an automatic thresholding conversion. The image analysis software was then used to count and measure the bright objects (fibrils) using the automatic analysis function. The width of the objects was reported and calibration was conducted by using the scale bar integrated with the individual SEM images. Image analysis was conducted on ~45 μm × ~55 μm images resulting in the identification of more than 750 individual fibrils for each image. The results of the automatic processing were confirmed by selecting ten fibril widths in higher

resolution images and manually measuring the fibril widths. The number-average degree of polymerization (DP_n) was calculated based on glucose monomer using BCA reducing end assay on freeze dried BC samples [14,15]. Each sample data point represents six separate pellicle isolates. (n=6)

Cellulase assays

The BC samples were evaluated for their susceptibility to enzymatic saccharification using the 2,4-dinitrosalicyclic acid (DNS) cellulase activity assay [16]. Standard curves were generated using glucose monomer. Each sample point represents three independently isolated pellicles (n=3). Whatman filter paper grades 1 and 42 served as controls (data not shown). Assay was adapted from Balsan *et al*. (2012) [17]. A total of 0.322 mg of cellulase from *Trichoderma reesei* ATCC 2691 (Sigma, St. Louis MO) was added to 20 mg of cellulose substrate (filter paper or bacterial cellulose) and 2 ml 0.2 M sodium acetate ($C_2H_3NaO_2$) buffer, pH 5.5. The mixture was incubated at 50ºC for one hour. Enzymatic activity was measured by monitoring the release of reducing sugars. Briefly, 1.5 ml of cellulose-enzyme solution was added to 3 mL of DNS reagent, boiled for 5 minutes, cooled to room temperature under running water, and the absorbance read at 540 nm (Wood and Bhat, 1988). Susceptibility of the BC to enzymatic saccharification was determined by amount of sugar released after a 20 min digestion period.

RESULTS AND DISCUSSION

Purity of BC

The effectiveness of the treatments described previously to provide pure native cellulose and remove secondary organic material, such as cell debris and media components, was first evaluated by ATR FT-IR shown in Fig. 1. Raised peaks are observed in 1637 cm^{-1} region and 2350 cm^{-1} region of NP-BC (Fig. 1a) corresponding to a primary amine group and CO_2 trapping, respectively. Additionally, a broader hydroxyl peak is observed at the 3,349 cm^{-1} peak region suggesting water association. Treatment of the pellicles with diH_2O (Fig. 2b) or 50 mM tris-HCl buffer (Fig. 2c) was effective at eliminating the CO_2 peak in the 2,350 cm^{-1} range, most likely due to the removal of cell debris. However, elevated peaks were still observed in the 1637 cm^{-1} region. The profile of PK-BC spectra (Fig. 2d) was nearly identical to BH-BC (Fig. 2e). The Avicel (microcrystalline cellulose) (Fig. 2f) spectra showed the complete elimination of the peak in the 1637 cm^{-1} region suggesting a slight difference in chemistry between commercial micro-crystalline cellulose and cellulose in more native states. It is

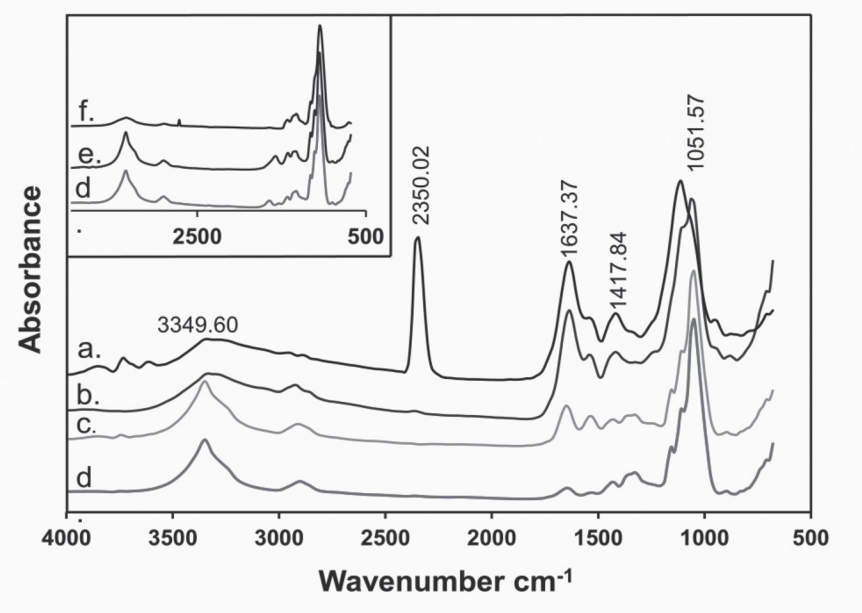

Figure 1. Normalized FTIR-ATR spectra of ground (a) Untreated BC [NP-BC] (b) diH_2O rinsed BC [Utr-BC] (c) 50 mM tris-HCl buffer rinsed BC [Tris-BC] (d) PK treated BC [PK-BC] (e) 1 M base treated BC [BH-BC] (f) Avicel (n = 6).

worth noting that the PK-BC and BH-BC cellulose have essentially equivalent spectrum indicating a comparable level of purity. To confirm these results, the PK-BC, BH-BC and Avicel were examined by FT-IR transmission (Fig. 2). The spectral profile of PK-BC was comparable to BH-BC and Avicel. The significant peaks related to cellulose were present across all regions. Although the magnitude of the varied by samples, this may be attributed to difference in prepping the sample for testing in the instrument. (The data presented above is based on standard error where n = 6.)

FT-IR ATR was an effective and efficient method of determining the purity of the BC pellicles. The respiration of *G. hansenii* results in the expulsion of CO_2, which provides buoyancy to synthesized pellicles [5]. This CO_2 peak is clearly evident in the untreated BC pellicles. The thorough rinsing of the BC pellicles removes the loosely associated bacteria and facilitates the release of CO_2 leading to the complete reduction in the CO_2 corresponding peak at 2,350 cm^{-1} [18]. The raised peak patterns in the 1,637 cm^{-1} region correspond to β-sheet and α-helical structures of proteins [19]. The addition of proteinase K results in the reduction of

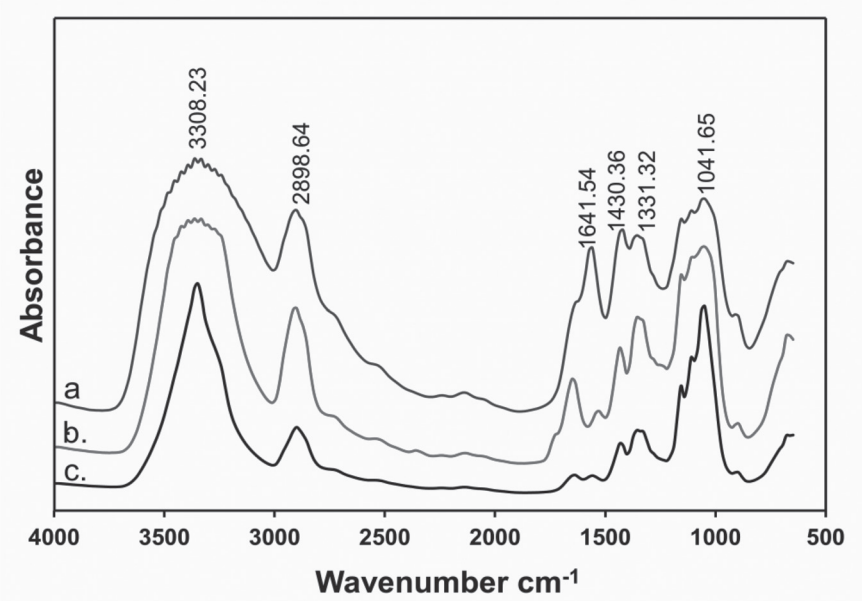

Figure 2. Normalized FTIR-transmission spectra of ground (a) 1 M base treated BC BH-BC (b) PK treated BC PK-BC (c) Avicel (n = 6).

these peaks, suggesting the complete removal of protein from cellulose pellicles. Cryogenically (liquid nitrogen) ground BC was used for transmission FT-IR analysis to attempt to provide increased resolution of BC chemical content. The pressed pellets of BH-BC, PK-BC and Avicel gave identical cellulose associated spectra in terms of peak locations. The deviations in the peak intensity are less informative as to materials present, but maybe indicative of artifacts resulting from sample preparation or slight differences in chemical composition. NP-BC and Utr-BC were also analyzed by FT-IR transmission. However, these pellets were sufficiently opaque to FT-IR that reliable spectra could not be obtained.

As a final evaluation of BC purity, an elemental analysis (Carbon and, Nitrogen) was performed on all BC pellicles and the results are represented in Table 2. Although the lowest percentage of nitrogen is observed in the BH-BC, the nitrogen content of PK-BC is an order of magnitude lower than either of the simply rinsed samples (diH_2O, tris-Buffer) or the untreated sample. The increased levels of nitrogen in the rinsed and untreated samples is a direct indication of the presences of cellular and extra-cellular proteins. In fact, it is plausible that some proteins remain on the cellulose as a result of incomplete removal of the proteins from the surface and intra-fibrillar spaces in the pellicles (n = 3).

Table 2. Bacterial Cellulose Elemental Analysis

Sample	*% Carbon*	*% Nitrogen*
PK treated BC (PK-BC)	43.16 ± 0.11	0.43 ± 0.02
1 M Base treated BC (BH-BC)	43.53 ± 0.04	0.31 ± 0.08
Untreated BC (NP-BC)	44.51 ± 0.13	4.68 ± 1.24
50 mM tris-HCl buffer rinsed (Tris-BC)	45.32 ± 0.24	3.27 ± 0.85
diH2O rinsed BC (Utr-BC)	31.88 ± 0.07	2.96 ± 0.96

Thermal characterization

To characterize the impact of our treatment on the thermal stability of BC, thermogravimetric analysis (TGA) was performed on untreated BC (NP-BC), 1 M base treat BC (BH-BC), and PK treated cellulose (PK-BC). Also for comparison, the thermogram was determined for Avicel. The thermograms in Fig. 3 clearly show a different behavior between the base treated bacterial cellulose and the PK treated bacterial cellulose. The untreated BC pellicle shows significant degradation over a wide range of temperatures. This is lost likely due to the degradation of proteins and other cellular debris that remains in the pellicle. This makes it difficult to discern the degradation of the cellulose from the other components. The PK treated cellulose shows significant less thermal stability of the cellulose

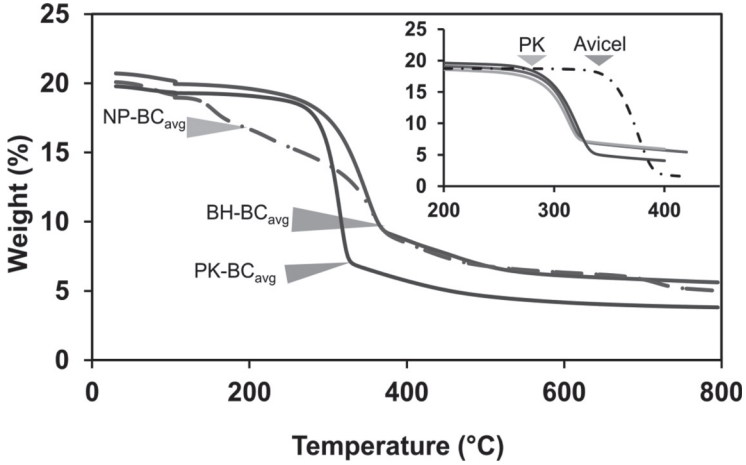

Figure 3. TGA Thermograms of Untreated BC NP-BC, 1 M base treated BC BH-BC and PK treated BC PK-BC and Avicel (n=3).

when compared to the 1 M base treated bacterial cellulose. Both the 1 M base treated and PK treated bacterial cellulose were significantly less stable when compared to the Avicel sample. Considering that the chemistry of all these samples were found to be substantially the same, the differences in the thermogravimetric properties can be attributed to either differences in the polymer structure (i.e. degree of polymerization), differences in the structure of amorphous and crystalline regions, or the possibility of chemical differences that were not detectable.

The differentials of the weight loss curves are shown in Fig. 4. The samples in Fig. 4 represent a systematic increase in severity of treatment. The mildest treatment being PK treatment, followed by PK plus boiling and then base treatment without boiling. Finally, the most severe treatment being base treatment and boiling. When the PK treated samples are boiled, the maximum degradation temperature shifts from 314ºC ± 1.45ºC to 341ºC ± 2.21ºC. The maximum degradation rate increases to 347ºC ± 3.75ºC from 326ºC ± 2.37ºC when the base treated sample is boiled. These results demonstrate the sensitively of the native cellulose structure to heat as well as chemical conditions. The lowered thermal stability of PK-BC compared to BH-BC and Avicel may be the result of the preservation of native cellulose structure (Cellulose I) [3]. Thermal stability of BH-BC has been enhanced by the addition of alkali. This result is consistent with what is observed in literature where an increase in degradation temperature to

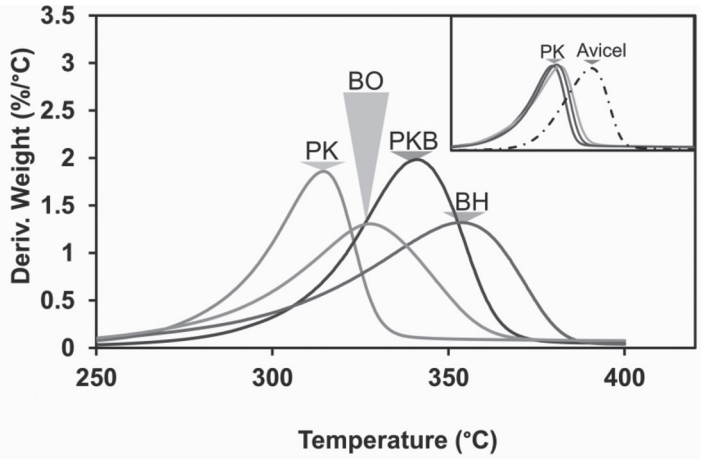

Figure 4. TGA thermogram first derivative weight % of PK-BC, base only treated BC (BO-BC), boiled PK treated BC (PKB) and BH-BC (n = 3).

360ºC is characteristic of cellulose II polymorphs [20,21]. Although the addition of alkali alone enhanced the thermal stability of BC, as seen in Fig. 4, the addition of heat to PK-BC produced a more dramatic change in thermal stability [3,20]. This suggests that the PK treatment works to a high degree to preserve the native state of bacterial cellulose.

To further understand the thermal properties of the cellulose samples, differential scanning calorimetry (DSC) was performed on all BC pellicles (Fig. 5). All samples exhibit a broad endothermic peak in the range of 100ºC, which can be putatively related to the loss of water from the hygroscopic materials. The untreated sample shows an endothermic peak in the range of 60ºC which can be attributed to the degradation of bonds within proteins and other transformation in the proteins. However, it is worth noting that the endothermic peak in the range of 100ºC is significantly different for the various samples. Comparing the 1 M base treated sample to the PK treated sample one can see the difference in thermal behavior below the degradation temperature. The PK treated sample exhibits an endothermic peak higher than the 1 M base treated samples. Based on the TGA results, which shows changes in the degradation behavior arising from boiling, the endothermic peak may be attributed to the rearrangement of the amorphous regions of the cellulose, changes in crystalline structure, or the possibility of an exothermic chemical reaction that stabilizes the cellulose. The TGA results (Fig. 3) does not show a significant difference in moisture content between the

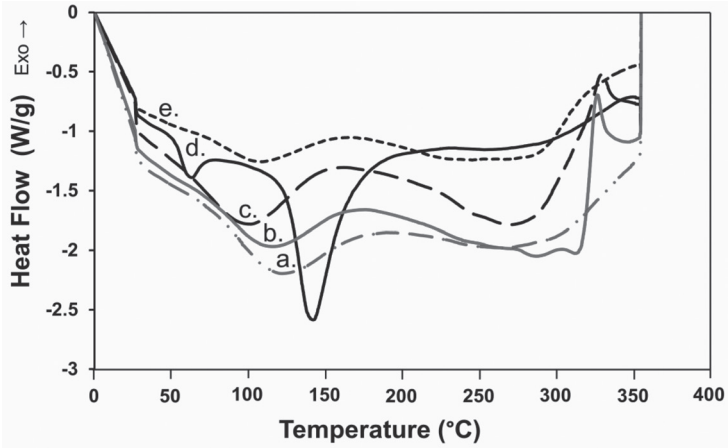

Figure 5. DSC Thermograms of ground (a) 1 M base treated BC BH-BC (b) PK treated BC PK-BC (c) diH_2O rinsed BC Utr-BC (d) Untreated BC NP-BC (e) 50mM tris-HCl buffer rinsed BC Tris-BC (n = 3).

two samples, which indicates that magnitude and shift in temperature cannot be simply attributed to water removal. The DSC thermograms also show differences in the degradation behavior of the two samples. The PK treated showed a greater endothermic peak when compared to the 1 M base treated sample.

Structural characterization

The crystalline index (C.I. value) of BH-BC and PK-BC was determined be XRD and amorphous region subtraction. The XRD spectra of the two samples are nearly identical. The similarity in the XRD spectra translates into similar values for the C.I. (Table 3). The significant difference the thermal degradation temperatures of the two samples cannot be related to the crystal allomorphs (i.e. same XRD) or the crystallinity (i.e. the more stable material has slightly lower C.I.) (Table 3) (Figs. 3 and 4)

The measured DP_n value for BH-BC was significantly higher than the DP_n value of PK-BC, 594 vs. 225. This difference in DP_n can be related to the differences observed in thermal stability [2]. It is observed that the increase in thermal stability is directly related to the increase in the DP. The increase in DP implies that cross-linking of the cellulose occurs and is related to mild heat treatment (boiling) in native cellulose. In the study performed by Kuo and Lee 2009, different acid and base associated pretreatments were investigated for the ability to influence the enzymatic saccharification of cotton based cellulose. The results

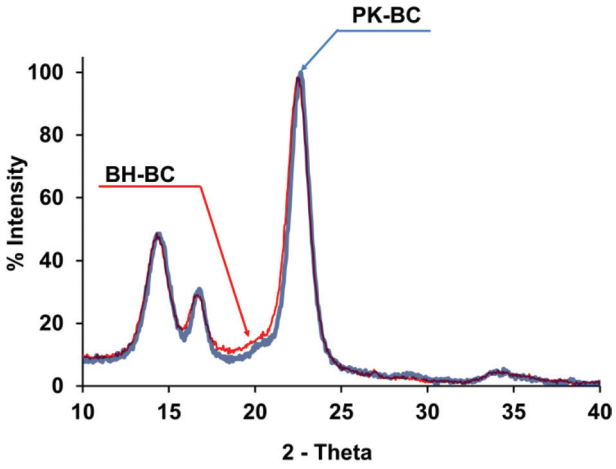

Figure 6. XRD spectra overlay comparison of (—) 1 M base treated BC [BH-BC] and (—) PK treated BC [PK-BC] (n = 3).

Table 3. Bacterial cellulose fiber physical properties post treatment

Samples	*C.I. value*	*DP_n Value*	*Cellulase Assay Conc. of D-Glucose mg ml^{-1}*
1 M Base Treated BC	81.86 ± 0.24	594.45 ± 5.08	2.23 ± 0.23
PK Treated BC	84.21 ± 0.54	225.61 ± 7.69	2.43 ± 0.25

C.I. (n = 3), DP_n (n =6), Cellulase assay (n = 3)

from this study illustrate the sensitivity of cellulose structure to different types of acids and bases resulting in significant changes in the biodegradability of the fibers. In this study, we build on the results presented in Kuo and Lee 2009 by analyzing the influence of chemical based pretreatments with and without heat on cellulose native structure. The results imply that even relatively mild treatments that only involve the addition of heat can have significant impacts on the native structure of cellulose. Furthermore, the addition of heat in the presence of low concentration base or acid can result in significant changes in the structural conformation of native cellulose in the physical properties of cellulose, resulting in a reduced saccharification efficiency. The rate of cellulose degradation via cellulose was measured. The PK treated and 1 % based treated samples differs by about 9 % with the PK bacterial cellulose having a slightly higher rate of degradation (Table 2).

SEM images presented in Fig. 7 provide insight into the effects of treatment on the fibrillar structure of the material. Figure 7a and 7b show the presences of cellular debris found frequently in the rinsed and untreated samples. Figure 7c and 7d show the differences between the 1 M base treated and PK treated samples. Observation of these images indicates that a difference in fibril dimensions exists. A set of lower resolution (larger area) images were used to provide a quantitative measure of the fibril dimension. The image analysis process is described in the experimental methods section. The length of the fibrils was not determined as the dimension often exceeded the area of inspection of the images (i.e. length typically greater than 55 μm). However, the width of the fibrils was determined, cf. Table 4. Table 4 shows a distinct difference in dimension between the two samples with 1 % base samples being 45 % wider than the PK treated samples. This confirms the visual inspection of Fig. 7 where there appears to be a a distinct difference in size.

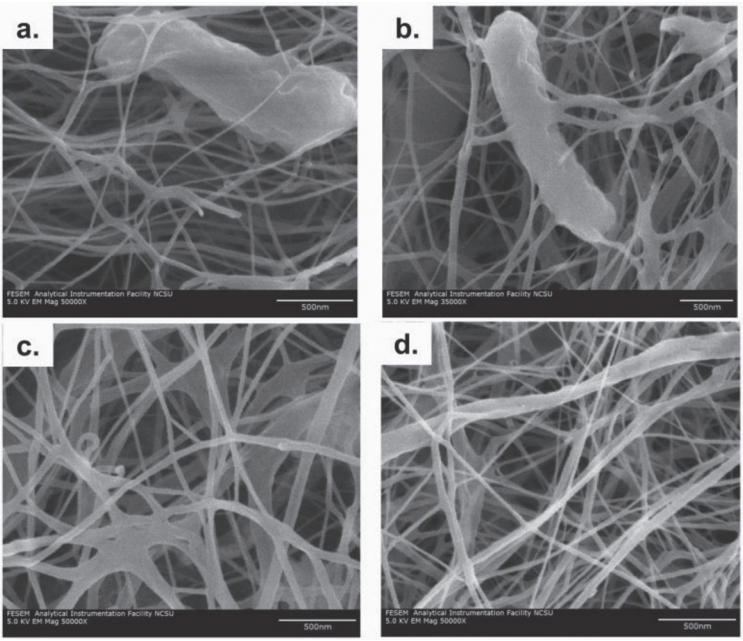

Figure 7. SEM images of (a) Untreated BC NP-NC (b) 50mM tris-HCl treated BC Tris-BC (c) 1 M base treated BC BH=BC (d) PK treated BC PK-BC.

Table 4. Average width of fibril

Samples	*Width Average (nm)*	*Standard Error (nm)*	*Sample Size (n)*
1 M Base Treated BC (BH-BC)	177	2	797
PK Treated BC (PK-BC)	122	0.4	1003

CONCLUSION

This work has significant implications in understanding the nature of the cellulose structure as it relates to many fields of application from papermaking to biofuels. This study shows the distinct effect that cellulose purification technique can have on altering the native structure of cellulose. Bacterial cellulose was isolated using a biologically compatible enzyme digestion method. By preventing the exposure to

heat, base, or acids, the observed structure and properties of the cellulose are altered. The mild enzymatic isolation technique showed that cellulose readily undergoes changes with heat associated pretreatments. This manifests itself in differences in the increased thermal stability of the cellulose and a lower temperature transition as observed in the DSC. XRD did not show significant differences in the crystalline structure (percent crystallinity or unit cell) suggesting that changes leading to the thermal stability take place outside of the crystalline regions. Furthermore, changes that take place during the heat and base treatment affect the degree of polymerization. Upon heat and base treatment, the DP of the cellulose doubles, which indicates a significant change in the material during this treatment. SEM images showed an increase in the fibril width with the addition of base and heat suggesting that the fibrils coalesce during this treatment. A 45 % increase in the fibril width was observed and found to be a significant increase in dimension. The changes in the fibrillar and DP of the cellulose lead to slight observed decline in the susceptibility of the fibers to enzymatic hydrolysis (9 %). In conclusion, this work shows that the native cellulose structure is much more susceptible to alteration by what is perceived to be mild chemical and heat treatment than previously understood. Thus, as researcher begin to attempt to understand the controlling factors for cellulose ultrastructure in cellulose biosynthesis, it will be critical to understand the artifacts that may arise due to isolation techniques.

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Transcription of Discussion

INVESTIGATION INTO THE STRUCTURAL AND THERMAL BEHAVIOR OF BACTERIAL CELLULOSE FIBERS AFTER BIOLOGICALLY RELEVANT PURIFICATION

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Bob Pelton McMaster University

The last couple of cellulose conferences I have been at, and there have been very impressive talks, people were talking about making body parts like blood veins out of bacterial cellulose. Looking at some of your images, these applications seem pretty scary. How can you possibly clean all the protein and other materials out of macroscopic chunks of the cellulose?

Joel Pawlak

That becomes a critical issue. We can take a lot of these proteins out but work that I did not present here (but will be publishing soon) tends to indicate that there are

proteins that remain in there. We believe that the extra-cellular proteins are extruded out of the bacteria and they actually are what is controlling our final cellulose structure. So yes, it is a bit scary, because there are some proteins and different things in there, but it is a good way to get funded, to create body parts.

Wolfgang Bauer Graz University of Technology (from the chair)

I have a question regarding the yield: how much bacterial cellulose per gram of bacteria is produced?

Joel Pawlak

There is a lot of variability in how you grow the cellulose and how you stress the bacteria. Depending on the concentration, you can probably get up to about 20 g L^{-1} for cellulose, but that depends on the culture conditions. There are some different ways to influence the growth rate, and we found out that part of the reason that the bacteria are producing the cellulose is actually as a defence mechanism. So if you apply antibiotics to the solution, it will produce more cellulose to help protect itself, so you can kind of trick it by stressing it. So that means, going back to Bob's point as well, antibiotics do not tend to kill these particular bacteria, they can persist through antibiotic attack due to the cellulose.

Bill Sampson University of Manchester

How did you measure fibre width?

Joel Pawlak

So for measuring fibre width, we have a number of different SEM images. The images in figure 7 in the text were not the particular images that we used, we used, I think, much bigger 20 μm × 20 μm SEM images, which we ran through an image analysis program that looked at the white objects in the images, and used an automatic algorithm to measure the fibre width. Then we went back and verified those measurements with a number of manual, individual measurements. So there is a little bit of trick in filtering to get rid of the fibres that are further way and just use the fibres that are more on the surface.

Bill Sampson

So are you applying any weighting to take into account, for example, the amount of fibre length that has a certain width? That might be a good thing to do.

Joel Pawlak

No. This is just a straight number average in this particular case.

Asaf Oko SP Technical Research Institute of Sweden

How do the bacteria actually form the network?

Joel Pawlak

Good question. We are not exactly sure how they are forming the network. In other words, do these bacteria become entangled in the network and then extrude the cellulose outwards, or do they extrude the cellulose whilst moving through the network? There is probably a combination of the two, and we think at some point in time, because we see them wrapped in cellulose in the network, that they become stabilized inside the pellicle itself. We have done some work where we look at where the different types of bacteria are, and there are actually some bacteria inside this medium that do not produce cellulose. So there are types of bacteria growing below the pellicle that are non-cellulose producers, but that is a biological question; there are some ideas on how they move, but it is not well understood.

Steve Keller Miami University

Is the cellulose resident as cellulose I or cellulose II; and is it in the α or β crystal form? If it is in the cellulose I form, would it be easily converted to the cellulose II form with alkali (mercerized), and what kind of structural and mechanical changes would you expect to observe in the new material?

Joel Pawlak

That's a good question but I won't speculate on cellulose I or II, α or β because, after doing this work, I am not sure there is such a thing. I think that the cellulose I α and cellulose I β structure that we are seeing might be artefacts of the purification technique at that point. So I think this kind of brings into question the overall cellulose structure. Most people say this is cellulose I α, but why are we getting the I α or I β structure? This is an interesting material because it is probably one of the few ways you can make a relatively large scale, nanofibrillar cellulose network that is non-bonded. So we can create very low density and strong structures, lower density than tissue densities, and putting them into different types of composites might be very interesting.

Discussion

Wolfgang Bauer Graz University of Technology (from the chair)

Did you also do solid state NMR tests on these different celluloses?

Joel Pawlak

We've been looking at this but, surprisingly in the US it is relatively difficult to get solid state NMR access where people know how to run it on cellulose. So we shipped some other samples over to Korea from the US to get them done, but we have not looked at these samples in detail yet, and we have not run this particular comparison yet.