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Production and characterization of bacterial cellulose before and after enzymatic hydrolysis

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Bacterial cellulose (BC) is produced by bacteria such as *Gluconacetobacter xylinus*. It has unique structural and mechanical properties and is highly pure as compared to plant cellulose. In this study, BC was produced in Hestrin-Schramm (HS) medium. *G. xylinus* produced an average dry yield of 1.4 ± 0.09 g/L BC after 9 days of fermentation period. Scanning Electron Microscopy (SEM) analysis confirmed BC to be highly structured while Fourier Transform infrared spectroscopy (FTIR) analysis indicated that the absorption peaks at $3000 - 3700$, $2800 - 2970$ and $1023 - 1024$ cm^{-1} were derived from -OH bending, -CH stretching and C-C/C-OH/C-H ring vibrations. X-Ray Diffraction (XRD) revealed a high purity of BC indicating type I cellulose with high crystalline nature. The solid state ^{13}C Nuclear magnetic resonance (NMR) spectroscopy of the untreated BC sample also indicated high crystallinity while the cellulase treated BC sample was different, but dominated by a polysaccharide signal between 55 and 110 ppm. The results suggest the prospects of the production and recycling of biopolymers from bacterial sources.

Key words: Bacterial cellulose, Scanning Electron Microscopy (SEM), Fourier Transform infrared spectroscopy (FTIR), X-Ray Diffraction (XRD), Nuclear magnetic resonance (NMR), cellulase enzyme.

INTRODUCTION

Cellulose is the most abundant macromolecule on earth and is one of the major components of the dry mass in materials such as cotton (90%) and wood (50%), (Hon, 1994; Keshk, 2014). The biosynthesis of cellulose is not exclusively found in plants alone but can be found in a variety of microorganisms such as algae and bacteria (Brown, 2004). Bacterial cellulose (BC) also known as microbial cellulose and bacterial nanocellulose is

produced by bacteria such as *Gluconacetobacter xylinus* (*G. xylinus*) (originally known as *Acetobacter xylinum*). *G. xylinus* acquires glucose and other organic substrates converting them into pure cellulose with unique structural and mechanical properties which are highly pure because of a lack of contaminating lignin and hemicellulose as compared to natural plant cellulose (Gayathry and Gopalswamy, 2014; Son et al., 2001). BC is secreted as

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an extracellular insoluble biofilm and forms a gelatinous mat on the air liquid interface due to its comparatively low density to water. Biosynthesis of BC requires a carbon source that is converted into Glucose-6-Phosphate, a metabolic intermediate for the formation of precursor substrate uridine diphosphoglucose (UDPGlc), which act as D-glucose donor to cellulase synthase to facilitate the polymerization of a β (1 \rightarrow 4) D-glucan chain (Rose et al., 1991). Many other carbon sources that are alternative to glucose have been reported in the production of BC by *G. xylinus* (Keshk, 2014; Mikkelsen et al., 2009; Sheykhnazari et al., 2011).

Cellulose synthase (CS) complexes are coded by the CS operon which codes for four different subunits, *bcsA*, *bcsB*, *bcsC* and *bcsD*; *bcsA* function in the acceleration of cellulose synthesis by combining with cyclic-di-GMP, while *bcsB* is involve in inner membrane attachment and the catalysing unit. Though the primary function of *bcsC* and *bcsD* are still not known, they are thought to be involved in the aggregation of each cellulose chain (Amano et al., 2005; Krystynowicz et al., 2005; Wong et al., 1990). Through extensive inter- and intra- fibrillar hydrogen bonding of the hydroxyl and esters groups, the sub-elementary fibre aggregates and crystallizes to form micro-fibrils (Hon, 1994; Ross et al., 1991). Depending on the formation and organization of the chain, cellulose is assembled in a parallel fashion known as type I cellulose which has high crystalline structural morphology with increased strength and stability, or into type II cellulose which is composed of antiparallel chains and results in an amorphous structure (Sarkar and Perez, 2012). Though many other microbes produce cellulose, those produced by *G. xylinus* have better quality and are more stable, and are also produced in a larger quantity (Ross et al., 1991). Intriguingly, *G. xylinus* strains which are known to be efficient producers of bacterial cellulose can also produce β -glucosidase. β -glucosidase is a rate limiting factor during enzymatic hydrolysis of cellulose; this is due to the fact that endoglucanase and exoglucanase which play an important role during cellulose hydrolysis are often inhibited by accumulation of cellobiose (Harhangi et al., 2002). Although these enzymes are known for their hydrolytic capabilities, they have been reported to play a role in the enhancement and production of BC (Koo et al., 1998; Standal et al., 1994; Tonouchi et al., 1995). β -glucosidases have the capability to catalyse and polymerise cellulose chains (Kono et al., 1999) and the encoding gene is thought to be located downstream of the cellulose synthase CS operon in *G. xylinus* (Tajima et al., 2001; Tonouchi et al., 1997).

This study provides for the first time a fully detailed characterisation of pure bacterial cellulose before and after treatment with a commercially available cellulase enzyme. The BC samples will be characterised by scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, Differentialscanningcalorimeter(DSC), Thermogravimetric

analysis or thermal gravimetric analysis (TGA) and Nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Microorganism

Gluconacetobacter xylinus 639 (*G. xylinus*) used in this study was obtained from the organisms collection centre of Microbiology laboratory, University of Wolverhampton, Wolverhampton United Kingdom.

Culture medium and growth conditions for BC production

The Schramm and Hestrin medium (HS medium) (Hestrin and Schramm, 1954) with initial pH adjusted to 6.0 using acetic acid was used to produce BC. HS medium was made up of (g/l): glucose – 20, bactopectone – 5, yeast extract – 5, disodiumphosphate – 2.7 and citric acid – 1.15. Inoculum was prepared by transferring two colonies of *G. xylinus* from an HS agar plate into a 250 ml conical flask containing 100 ml sterile HS medium. The culture was incubated at 25°C on a water rotary shaker for 24 h at 150 rpm. After 24 h, the cellulose pellicle formed on the surface of the culture broth was vigorously shaken in order to remove active cells embedded in the cellulose membrane. 6 ml of the cell suspension was introduced into 94 ml of fresh SH medium. The culture was cultivated statically at 30°C for 9 days. The synthesized cellulose was harvested and then purified by boiling in 1% NaOH for 2 h and next it was thoroughly washed in tap water until the product was transparent while the second sample was washed with distilled water only.

Cellulase pretreatment of BC

Cellulase C1184 from *Aspergillus niger* (Sigma-Aldrich, UK) which catalyzes the hydrolysis of endo-1,4- β -D-glycosidic linkages in cellulose was used in the hydrolysis of the BC. Five gram (5 g) BC was incubated for 12 h with 2 mg/ml cellulase enzyme at 37°C. To inactivate the enzyme adsorbed to the BC, 1 M NaOH (pH 13.3) was used to treat the hydrolysed BC for 10 minutes. The enzymatically pre-treated BC was then thoroughly washed with distilled water until pH 7.2 was achieved. The treated BC sample was freeze dried and stored at room temperature for further analysis.

Characterization of bacterial cellulose

To study conformational characteristics, BC polymer was characterized using microscopic and spectroscopic methods. The microscopy method used was SEM. The spectroscopy techniques used included FTIR, XRD, DSC and Thermogravimetric analysis or thermal gravimetric analysis (TGA).

Scanning electron microscopy (SEM)

The BC fibril (cellulase treated and untreated BC) was characterized using SEM (Zeiss Evo 50 fitted with an Oxford EDX, Zeiss, UK). Scanning electron microscopy reveals information about the BC chemical composition, crystalline structure and the external morphology of the sample. A thin layer of freeze dried BC was gold coated using an ion sputter-coater to improve the conductivity of the samples and the quality of the SEM images (emscope SC 500).

The gold coated sample was viewed and the images were digitally captured.

Fourier transform infrared spectroscopy (FTIR)

To study conformational characteristics of BC obtained from HS medium, polymer was analysed by FTIR spectrometer (Genesis II with DuraScope, Mattson Instruments UK) in a transmittance mode at wavelength ranging from 4000 to 400 cm^{-1} . The FTIR provided information on chemical structures and physical characteristics of the BC produced. BC samples were initially freeze dried and made into powder. A little quantity of the sample (or cellulase treated BC), just enough to cover the disc hole was placed on the disc. The disc was pressed and the scan of BC was operated on the personal computer (PC) which further generates the IR spectrum. A detector monitors the wavelength range and transmits the signal to a computer which translates the signal into an absorption spectrum. A standard cellulose material C6288 (Sigma Aldrich UK) was used as a reference.

X-Ray diffraction (XRD)

X-Ray diffraction (XRD) is a rapid analytical technique that is used in the qualitative identification of crystalline phases by their diffraction pattern. In this study, XRD analysis was utilised to characterize the crystallinity index and the degree of polymerisation of the BC sample. This was performed using the Empyrean PANalytical diffractometer (Philips model PW1770) at the University of Wolverhampton. BC was ground and homogenised before analysis. The sample was then placed in the sampling tray where the surface was smoothed to eliminate surface irregularities. The X-ray diffraction spectra were recorded using an Empyrean PANalytical diffractometer system in steps of 0.1° using Cu K α radiation as X-ray source at 25°C . X-ray diffraction spectra were recorded using diffractometer at a plate current intensity of 40 mA and an accelerating value of 40 kV. Scans were performed over the $5.0064 - 79.9904$ [2θ] range using step 0.0130 in width.

Solid state nuclear magnetic resonance (SSNMR) spectroscopy

Solid-state NMR spectra were obtained at the EPSRC UK, National solid-state NMR service at Durham. Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique that is used to determine the content and purity of a sample as well as its molecular structure. The solid state ^{13}C NMR spectra were measured on a Varian VNMRS 600 spectrometer. When untreated and cellulase treated BC samples were irradiated with a radio frequency, absorption of the resonant energy between adjacent energy levels occurs. The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy was emitted at the same frequency. The precise frequencies at which the spin-active BC resonates was picked up and displayed by the NMR spectrometer.

Differential scanning calorimeter (DSC)

DSC is a thermos-analytical technique used to study what happens to polymers when they are heated, that is thermal transitions of a polymer. Two aluminium pans are used; sample is placed in one pan while the second pan which serves as a reference pan is left empty. The two pans are heated at specific rate, say 20°C per minute. For untreated BC, 8.2220 mg of BC from the compression moulded products was loaded into aluminium pans. DSC

measurement were taken with a TA-DSC 2010 apparatus (TA Instruments, Newcastle, DE, USA), under the nitrogen atmosphere (flow = 50 mL/min). The instrument was calibrated with high purity indium. The melting temperature (T_m) was taken as the peak temperature maximum of melting endotherm. In this study, T_g was taken as the midpoint of the step-transition.

A first temperature program was conducted to record T_g and T_m . After heating to 200°C at $20^\circ\text{C}/\text{min}$ in order to erase thermal histories the BC samples were cooled down to -100°C at a cooling rate of $30^\circ\text{C}/\text{min}$. During the first heating run the broad endotherm could probably be connected with solvent or water was observed. A second heating scan was conducted from -70 to 280°C at $20^\circ\text{C}/\text{min}$ and two thermal transitions were recorded. The third heating scan was conducted again from $-30 - 3500^\circ\text{C}$ at $20^\circ\text{C}/\text{min}$ and melting transition at 103.21°C and T_g at 38°C was recorded. For cellulase treated BC, 14.2590 mg BC was used and the same procedure was carried out as described above with only first and second scan rounds using same conditions.

Thermogravimetric analysis or thermal gravimetric analysis (TGA)

TGA is a thermos-analytical technique in which changes in weight are measured as a function of increasing temperatures. To determine the thermal stability of untreated and cellulase treated BC, approximately 3,4830 mg of the untreated BC and 5,6700 mg of the cellulase treated BC were placed in aluminium pans. Raw and derivative weight data were used to determine decomposition, temperatures and associated weight losses. TGA analysis was performed with a TGA/DSC1 Mettler-Toledo thermal analyser at a heating rate of $10^\circ\text{C}/\text{min}$ in a stream of nitrogen (60 mL/min). The obtained TGA data were analysed using the Mettler-Toledo star system SW 9.30.

RESULTS

In this study, BC was obtained as a thick layer from HS medium and the average wet yield of cellulose by *G. xylinus* was found to be 90.48 ± 12.14 g/l with an average dry yield of 1.4 ± 0.09 g/l. The BC samples used for analysis were harvested from a single flask of HS media containing multiple layers of BC (Figure 1). The multiple layers of BC were thought to be caused by the formation of a new pellicle due to the preceding pellicle sinking.

This unique opportunity of formation of new pellicle due to preceding pellicle sinking eliminates much variability that can affect the quality of the BC thus allowing a better comparison among samples. All samples were similar in appearance and thickness. Samples were processed with distilled water (dH_2O), $\text{dH}_2\text{O} + \text{NaOH}$, cellulase pretreatment and were freeze dried before the IR analysis.

Fourier Transform infrared spectroscopy (FTIR) Analysis

Table 1 is a summary table of corresponding wave numbers derived from the FTIR analysis associated with interpreted bonds and functional groups (Dai and Fan, 2010; Fan et al., 2012; Nam et al., 2011).

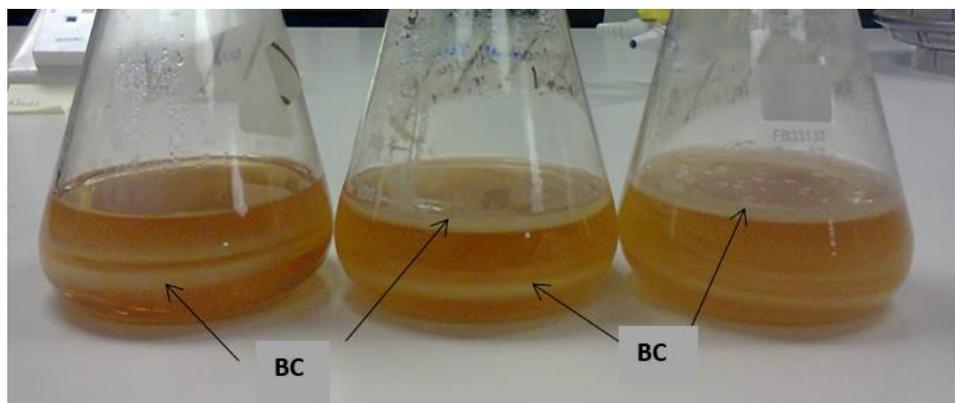


Figure 1. Layers of bacterial cellulose formed by *G. xylinus* 639 in triplicate.

Table 1. Summary table of corresponding wave numbers derived from the FTIR analysis associated with interpreted bonds and functional groups (Dai and Fan, 2010; Fan et al., 2012; Nam et al., 2011).

Wavenumber peaks (cm^{-1})	Type	Type of vibration	Functional group
3328	Broad	Stretching	-OH (H-bonded)
2950	Narrow	Stretching	C-H
1620	Narrow	Bending	-OH of absorbed water
1380		bending	Planar CH
1153		Stretching	Asymmetrical C-O-C
1020		vibration	C-C, C-OH, C-H ring and side group

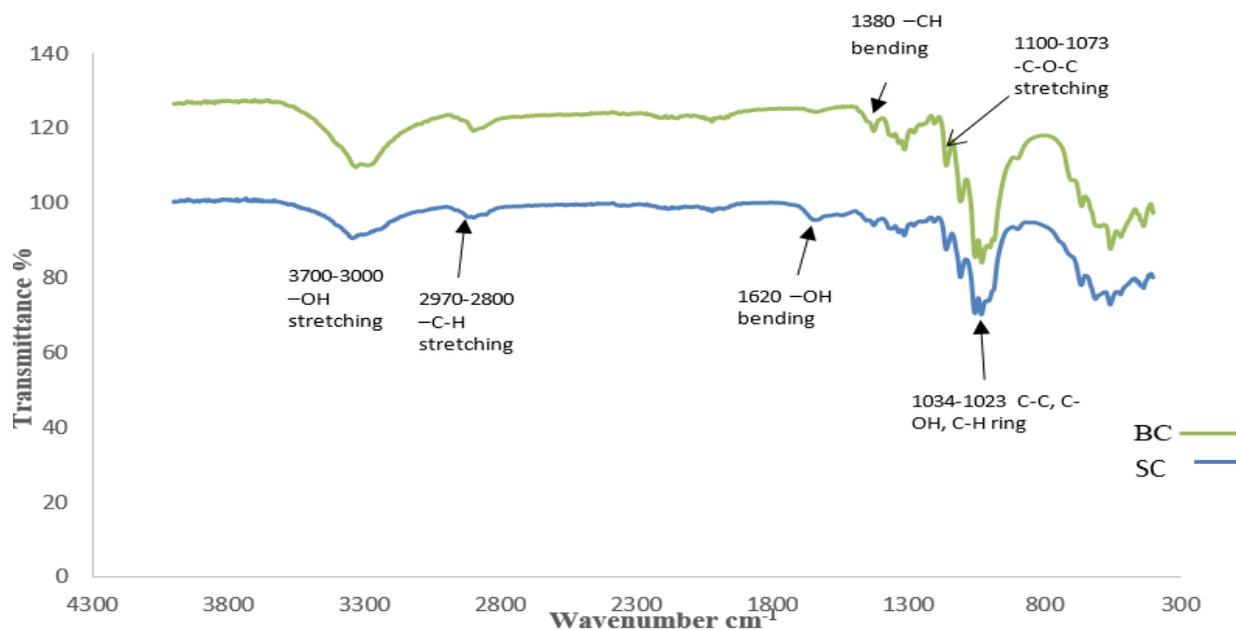


Figure 2. FTIR spectrum of freeze-dried BC and standard cellulose. SC: Standard cellulose (Sigma-Aldrich, UK), BC: Bacterial cellulose.

Figure 2 shows the FTIR spectra in $4300\text{-}300\text{ cm}^{-1}$ region of BC and standard cellulose from Sigma-Aldrich.

Both diffraction curves are of typical cellulose structure with a few exceptions of the BC spectra from the

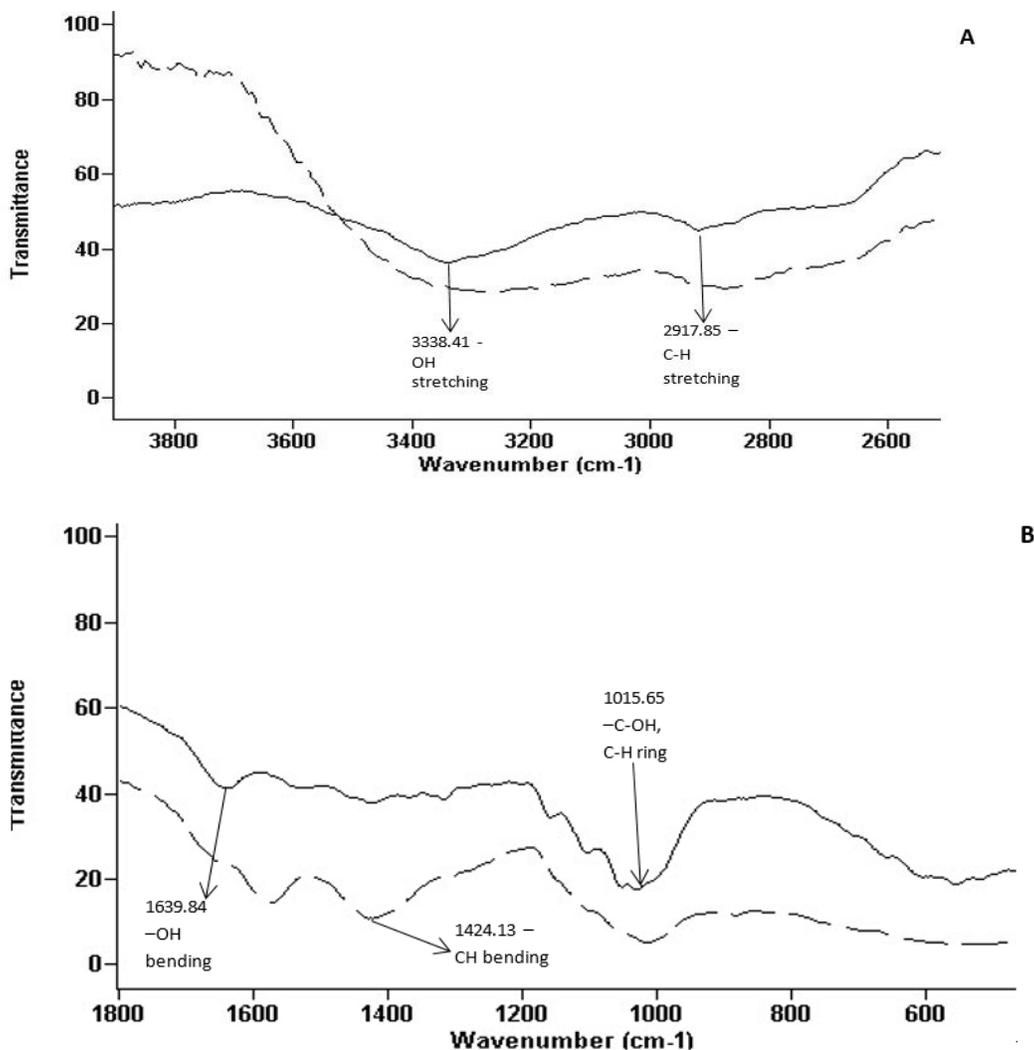


Figure 3. FTIR spectrum of BC before and after pretreatment with cellulases. A: 3800 – 2600 cm⁻¹ region; B: 1800 – 600 cm⁻¹ region. - - - - Treated BC, Untreated BC.

standard cellulose spectra. FTIR spectra in 1620 - 1380 cm⁻¹ region show the band of the NaOH washed BC to be shifted. The bands at 37000 - 3000, 2970 - 2800, 1100 - 1073 and 1034 - 1023 cm⁻¹ are similar.

Figure 3 (A and B) show the FTIR spectra in 3800 – 600 cm⁻¹ region of BC and cellulase hydrolyzed BC which were employed to characterize the structure of the BC material before and after treatment with cellulase enzyme. The spectra shown in Figure 3A show the absorption spectra band at 3338.41 and 2917.85 cm⁻¹ for –OH stretching and –C-H stretching respectively. The absorption spectra band at 1015.65 cm⁻¹ (Figure 3B) is assigned as C-C, C-OH, C-H ring and C-O-C stretching at the β-(1→4)-glycosidic bond/linkage in cellulose (Nelson and O'Connor, 1964) while the absorption spectra band at 1424.13 cm⁻¹ is assigned to the CH₂ scissoring motion in cellulose (Spiridon et al., 2011). Similarly, the absorption spectra band at 1639.84 cm⁻¹ is

assigned to the –OH bending. As shown in Figure 3B, the absorption spectra band at 600 - 1000 cm⁻¹ was strong for untreated BC but somehow weak for the treated BC material.

SEM analysis

SEM analysis was carried out with 4 different samples, of BC after freeze drying. Figure 4A show the SEM micrographs of unwashed BC microfibrils and the bacterial cells enmeshed in it. Figure 4B is SEM images of threadlike cellulosic microfibrils without visible bacterial cells. The fibrils are tightly packed with dendritic nodes present which are thought to be regions of amorphous cellulose (Liu et al., 2013). The tightly packed fibrils conferred morphological features similar to pure microcrystalline cellulose. There were also regions of

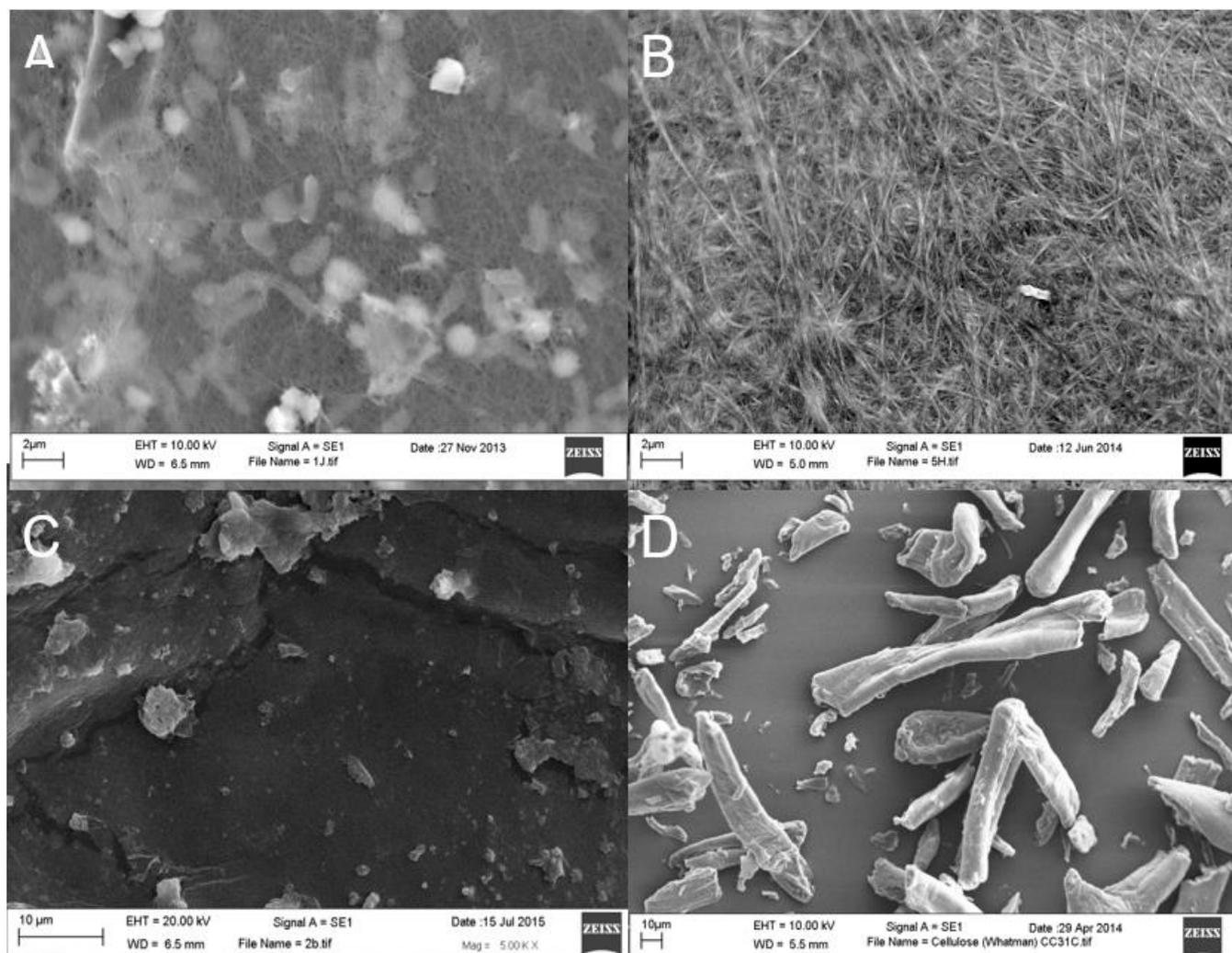


Figure 4. SEM collection of BC micrograph. A: Rod-shaped cells of *G. xylinus* (approx 1.5-2 μm in length) entangled in BC, B: Cellulosic fibrils of BC, C: Cellulase treated BC (2 mg/ml), D: The non-microbial cellulose sample from Sigma-Aldrich.

highly crystallized cellulose which contained fibrils that are more defined and orientated. Figure 4C show the SEM images of cellulase treated BC that was hydrolyzed to sugar monomers. The micrograph shows that some microfibrils are separated while others cluster together. Figure 4D show the SEM image of the non-microbial cellulose sample (Sigma-Aldrich) which differs considerably from the BC; and exhibits a discontinuous and fragmented formation. The non-microbial cellulose shows cellulose sheets having large gaps between the fibers and the fibers are not intertwined closely, while the BC has much smaller fibers which are closely intertwined.

XRD analysis

A typical X-ray diffractogram obtained from the BC sample demonstrates two characteristic clearly resolved

peaks (Figure 5). The crystallinity index was estimated based on the diffractogram. X-ray diffractogram investigations indicated that majority of the cellulose was type-1 cellulose.

Solid state nuclear magnetic resonance (SSNMR) spectroscopy

To determine the content and purity of untreated and cellulase treated BC, solid state ^{13}C NMR was carried out using Varian VNMRS 600 spectrometer. Figure 6 shows the spectrum from an untreated BC sample. The BC untreated is dominated by a set of signals consistent with cellulose. Judging from the intensity of 89 ppm peak to the broader one at 84 ppm, the BC is quite highly crystalline.

Figure 7 shows the spectrum of cellulase treated BC

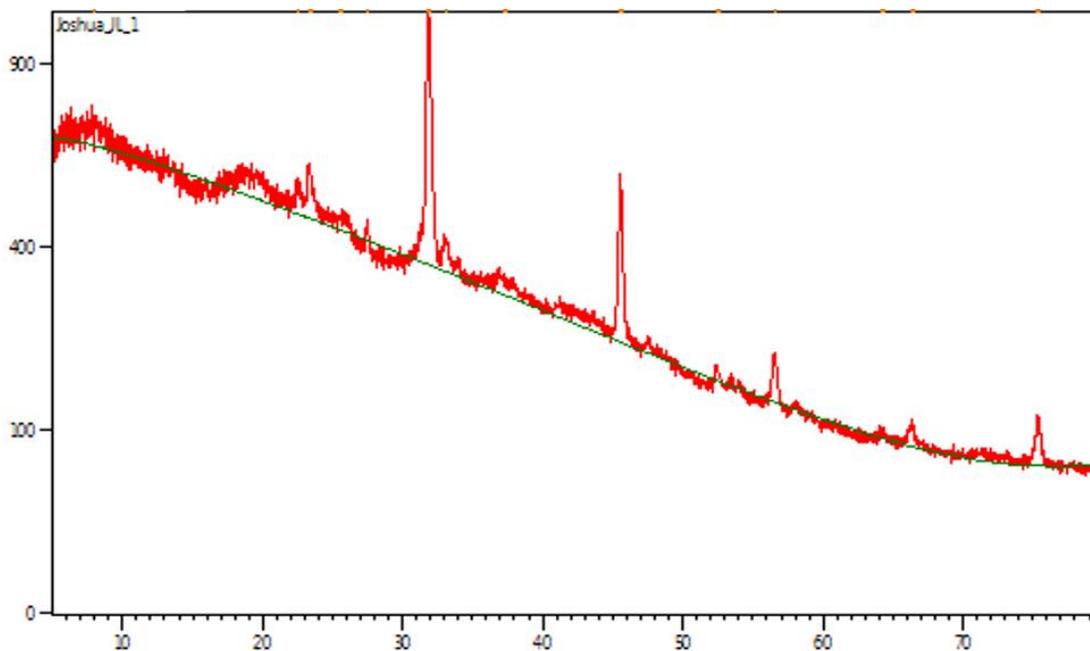


Figure 5. XRD diffraction result of untreated bacterial cellulose (BC).

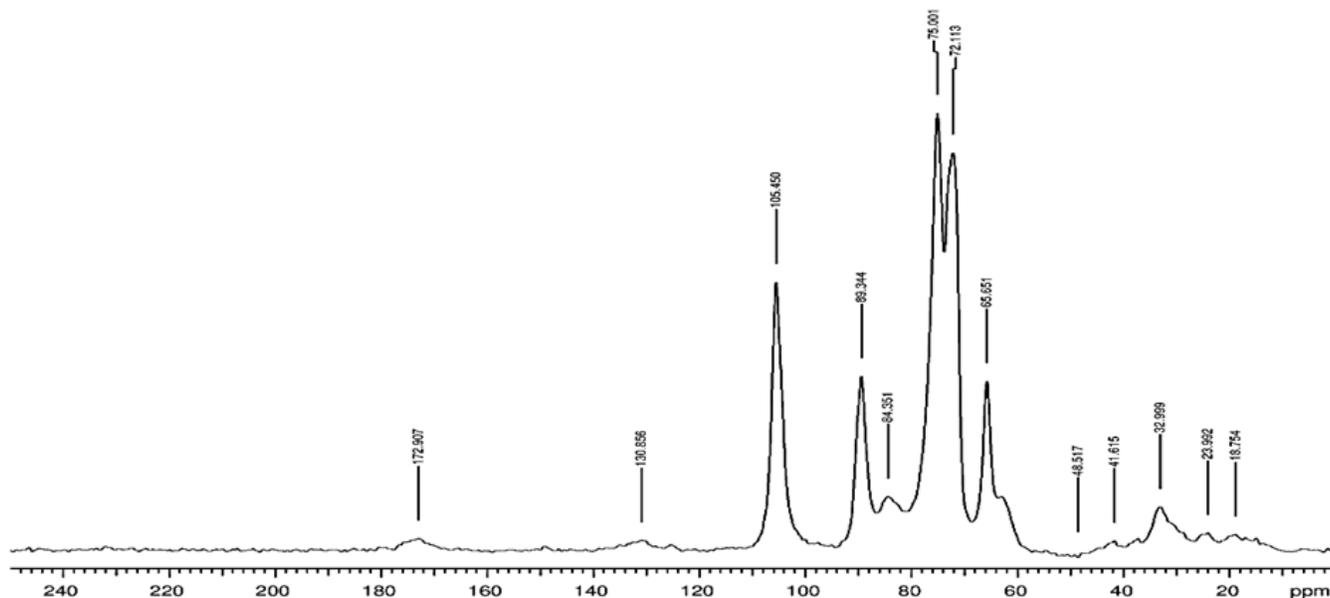


Figure 6. ^{13}C SSNMR spectrum of untreated bacterial cellulose (BC).

sample which is different from the untreated BC. The spectrum is still dominated by the polysaccharide signal between 55 and 110 ppm but it is much less well defined than for the untreated BC sample. The spectrum also show some other signal at the low-frequency end of the spectrum and four sharp lines at the high frequency end that were not present in the untreated BC sample.

Differential scanning calorimeter (DSC)/Thermogravimetric analysis (TGA)

To determine the thermal transitions of untreated and cellulase treated BC, glass transition (T_g) and melting temperature (T_m) were evaluated. The DSC of the untreated BC indicated that the T_g and T_m were around

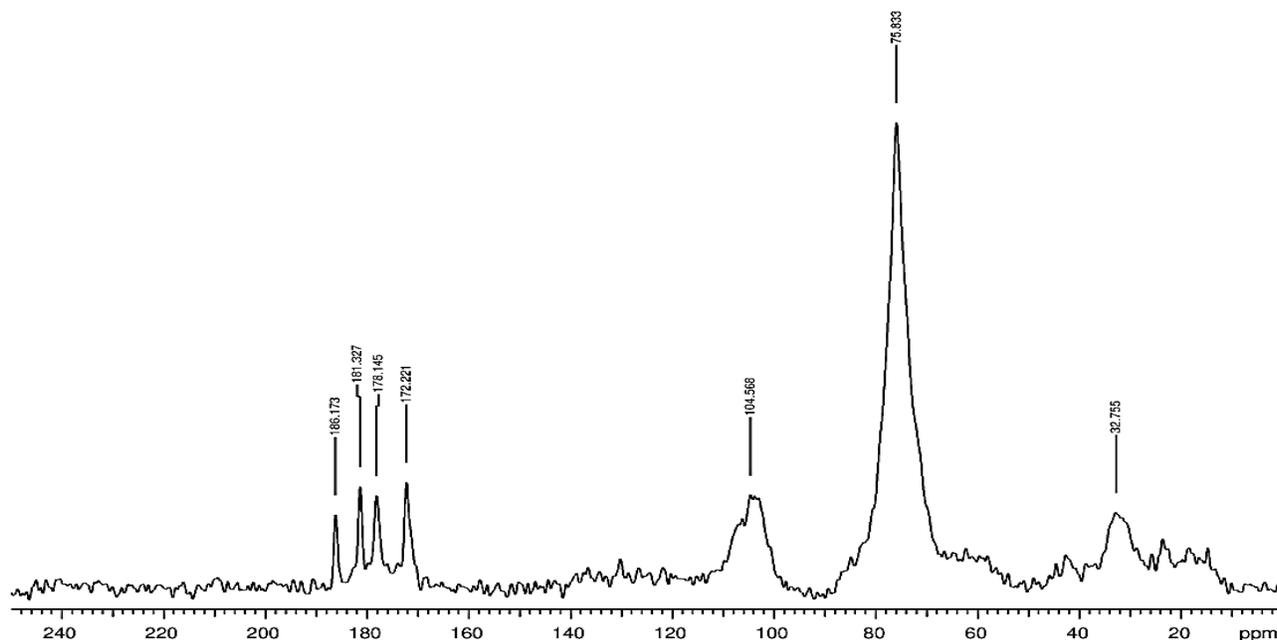


Figure 7. ¹³C SSNMR spectrum of cellulase treated bacterial cellulose (BC).

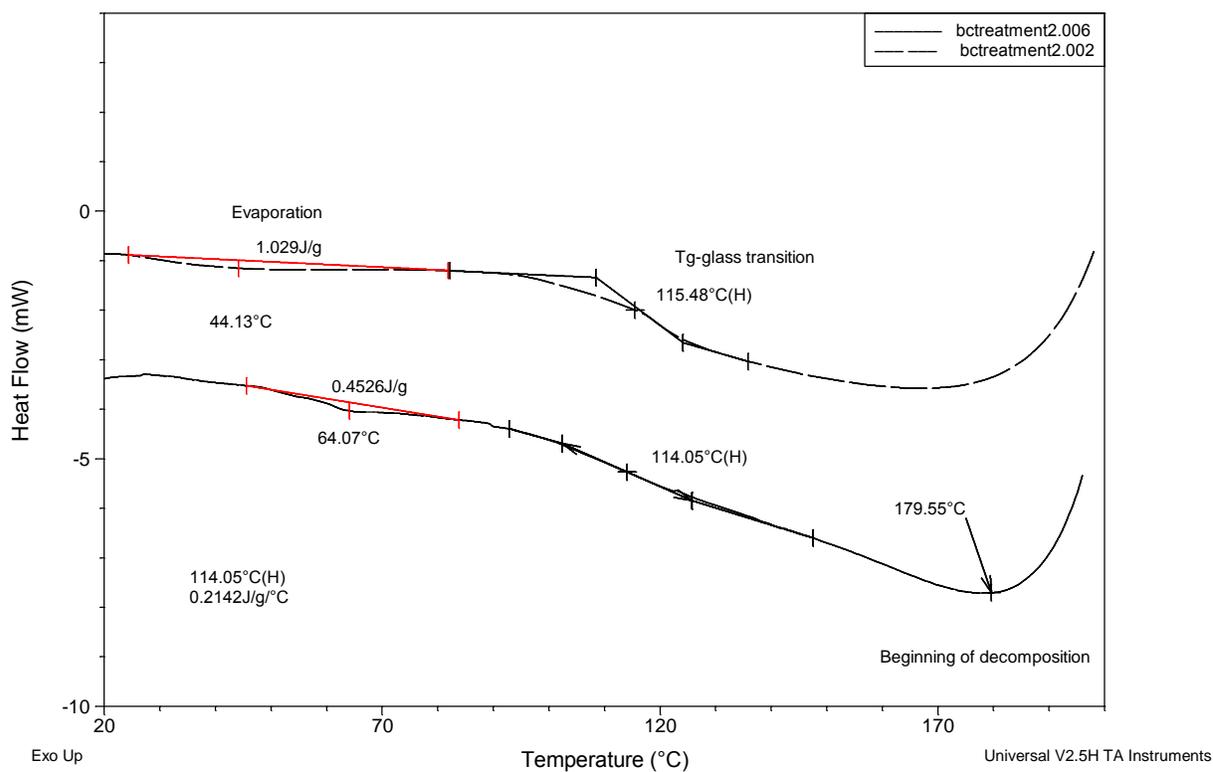


Figure 8. DSC thermogram of cellulase treated BC illustrating the determination of Tg and Tm.

38 and 117.31°C respectively (Figure 8). The BC sample was stable up to 200°C above which decomposition

started with a pronounced disintegration at 338.13°C. The initial BC sample varied greatly between 10 to 200°C

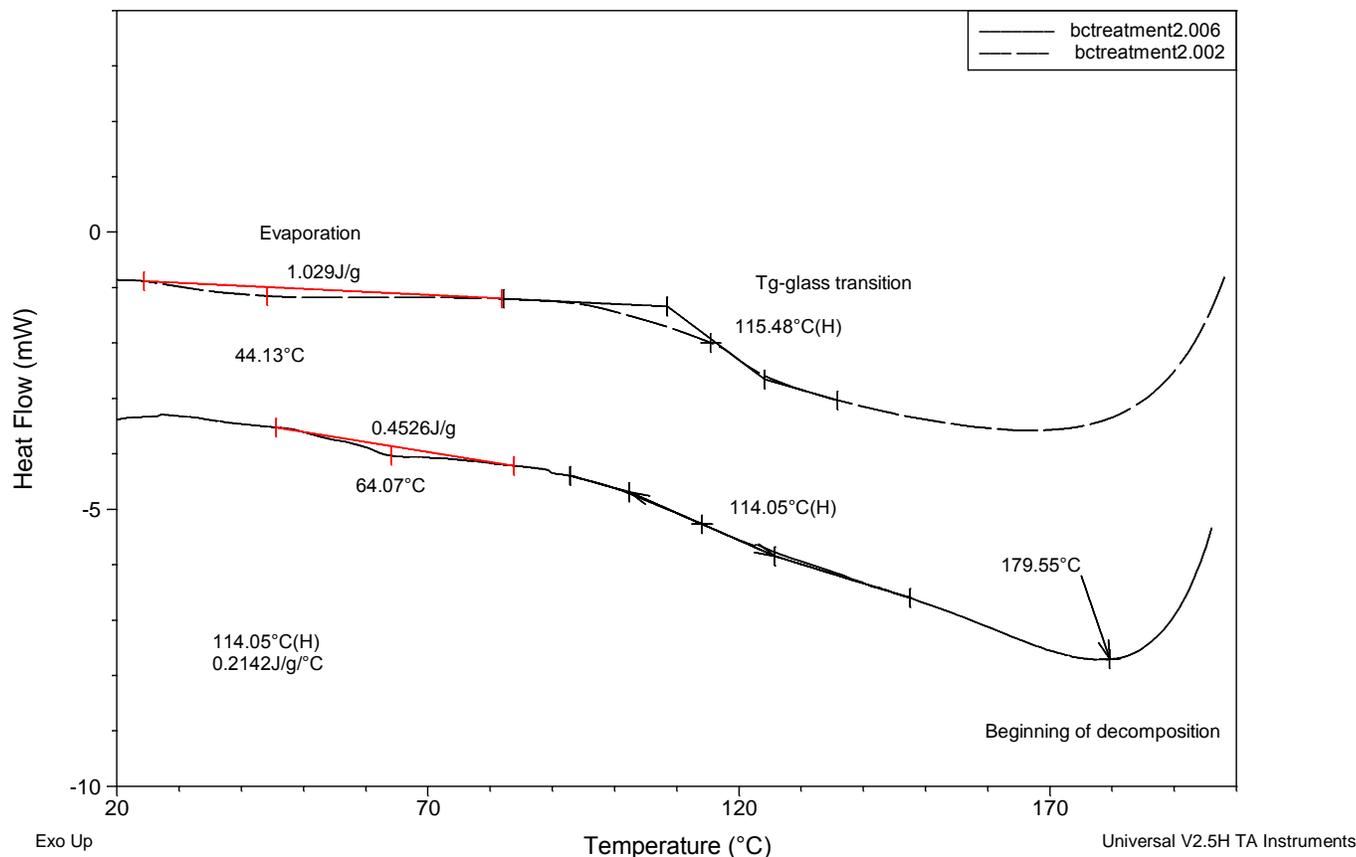


Figure 9. DSC thermogram of cellulase treated BC illustrating the determination of Tg and Tm.

as a result of evaporation due to water contents. On the other hand, the Tg of cellulase treated BC samples were around 115°C (Figure 9). Melting transition was not observed. A small endotherm probably due to solvent evaporation was observed at 44-60°C. There was quick evaporation of the sample which was stable up to around 150°C above which disintegration began and there was pronounced decomposition at 170°C.

The TGA curve for untreated BC sample shows bound water for the untreated BC sample to be evaporated at approximately 50°C with degradation temperature after 200°C (data not shown). The TGA of the cellulase treated sample shows a fast evaporation of the sample at a temperature of 30-80°C which could be ascribed to the presence of moisture and other materials used in the treatment of BC sample. The treated sample was stable until about 150°C before degradation. The maximum mass loss for the cellulase treated BC sample was much higher than that of the untreated BC sample.

DISCUSSION

After nine days of static fermentation, bacterial cellulose

(BC) was obtained as a thick layer on the surface of HS medium. The dry yield of BC produced by *G. xylinus* was found to be 1.4 ± 0.09 g/L. The thickness of the wet BC was 12 mm and the thickness of the dry sheets was 0.18 mm. The yield results of BC production in this study is lower than the previous work of Carreira et al. (2011) and Gayathry and Gopalswamy (2014) who reported BC production with a weight of 2.60 and 11 g/L respectively. Differences in yield may reflect strain differences.

Figure 2 shows the FTIR absorption spectrum of standard cellulose and BC. The FTIR results as deduced from Table 1 indicated many similarities between the bacterial cellulose and the standard cellulose. Results indicated that all samples contain functional groups that were associated with cellulose, such as: $3700-3000\text{ cm}^{-1}$ hydrogen bonding -OH bending, $2970-2800\text{ cm}^{-1}$ -CH symmetrical stretching and $1034 - 1023\text{ cm}^{-1}$ C-C, C-OH and C-H ring side group vibrations, which is typically widely reported for bacterial cellulose (Fan et al., 2012; Trovatti et al., 2011).

The broad absorption spectra in the region $3600 - 3000\text{ cm}^{-1}$ (-OH stretching, H-bonded) indicates a shallower peak for the treated BC sample. Treatment with cellulase enzyme is thought to disrupt the $\beta(1\rightarrow4)$ linkages

between the cellulose chains (Fan et al., 2012). Hydrolysis of the β -linkages can lead to changes in the molecular, supramolecular and morphological level orientation (Fan et al., 2012) which can have a variety of effects on the physical properties of the BC such as crystallinity, water holding capacity and mechanical strengths. The spectra in Figures 2 and 3 (A and B) indicate that the untreated BC possess type I cellulose. The type I cellulose was transformed into type II cellulose or amorphous cellulose after enzymatic hydrolysis (Figure 3A and B).

The -OH bending of adsorbed water was observed at 1639.84 cm^{-1} for untreated BC sample. This result is in agreement with those previously reported (Okiyama et al., 1992) whereby 0.3% of the 90% of water (by mass) is held through the extensive hydrogen bonding network facilitated by the hydroxyl groups and ester bonds. In untreated BC samples, the intensity for adsorbed water is comparably less intense than those found in cellulase treated BC sample spectra. The FTIR absorption spectra for both treated and untreated BC show characteristic peaks around $1000 - 1200\text{ cm}^{-1}$ (Langkilde and Svantesson, 1995; Zhibankov et al., 2000) and the band around 1424.13 cm^{-1} in treated BC can be ascribed to CH shake vibrations in cellulose (Spiridon et al., 2011). Generally, the results of the FTIR investigation in Figure 3A and B showed that the peaks of BC samples were lower after enzymatic hydrolysis indicating that the BC was degraded. The band at $1400-1600\text{ cm}^{-1}$ for the treated BC sample could be attributed to the absorbed water bending vibrations after enzymatic hydrolysis.

Confirmation of the general structure of the untreated and cellulase treated BC was carried out using the SEM analysis. BC images from SEM micrographs (Figure 4) revealed complex meshed cellulose ribbons that interweaved among each other to form the BC membrane. Figure 4A is an SEM image of BC washed with dH_2O showing distinct rod-shaped cells that were immobilized and entangled by the fine cellulose ribbons. The length of each cell is roughly 1 to 2 μm . The regions containing thicker cellulose ribbons displayed no obvious sign of cells, which suggests that the cells were covered by the successive formation of cellulose and that these fine fibrils were in the early stages of cellulose matrix formation. The absence of other cell types indicates that *G. xylinus* is the only producer of the BC pellicle. Interestingly, samples that were subjected to repeated and prolonged washing with dH_2O showed no presence of cells within their matrix thus suggesting that thorough washing of BC with dH_2O is effective in removing cells and other cellular debris.

Figure 4B shows the SEM image of threadlike cellulosic microfibrils of BC. White dendritic nodules were observed throughout the structure of the BC, forming junctions, diverging microfibrils from the center which were then incorporated to the surrounding meshwork. The dendritic nodes which were widespread throughout the sample in a

variety of shape and sizes were amorphous in nature indicating the presence of type II cellulose (Sarkar and Perez, 2012). Whilst washing of BC with dH_2O was effective in removing cells, treatment of the sample with NaOH before washing with dH_2O may be successful in the context of sterility. A dramatic morphological change on BC microfibrils was observed after hydrolysis (Figure 4C) indicating that BC microfibrils were hydrolyzed to sugar monomers.

The XRD analysis result gave an insight into the physical properties of the BC produced. The bacterial cellulose diffractogram reveals two principal diffraction peaks at 15° and 29° confirming the presence of type-1 cellulose. Previous studies indicated that a high intensity at diffraction plane 14.5° and 22.6° indicates the presence of cellulose type-1 (Czaja et al., 2004; Moosavi-Nasab and Yousefi, 2011; Sheykhnazari et al., 2011).

The ^{13}C SSNMR spectrum of untreated BC is presented in Figure 6. The broader peak was due to the crystallite surfaces and the amorphous domains while the comparatively sharp peak was due to a crystalline region (Shezad et al., 2010). The NMR spectrum of the BC is in agreement with those reported in the literature for BC and cellulose (Shezad et al., 2010; Zhao et al., 2007). Although the SEM analysis (Figure 4 C) show a dramatic changes on cellulose microfibrils of cellulase treated BC sample, the NMR data (Figure 7) were still dominated by polysaccharide signals. The NMR result of the cellulase treated BC show that BC apparent crystallinity was alternated by hydrolysis which suggests that BC could be a valuable raw material for fermentable sugars.

The thermal behaviour evaluated by DSC and TGA shows the thermal stability of BC. DSC measures the heat absorbed or released by a material as a function of temperature or time. The Tg of the untreated BC was found to around 37.14°C which was found to be lower than the value 44.28°C reported by Mohite and Patel (2014) but higher than the value of 13.94°C reported by George et al. (2005). The higher the Tg of a material, the better the advantage because minimal aging is expected at a storage temperature which is below Tg (Bechard et al., 1995). The thermal stability of BC up to 200°C could be attributed to its crystallinity and high molecular weight (Chen et al., 2009) while the low thermal stability of treated BC could be as a result of enzyme hydrolysis yielding low molecular weight oligosaccharides. The results indicated that the cellulase treated BC sample was hydrolysed hence the low Tm and decomposition temperatures compared to the untreated BC samples.

Thermal degradation temperature of a sample is influenced by the following factors: sample size, moisture content of sample and the stability of functional groups. Other features include experimental factors such as nitrogen flow rate and the heating rate (Chang et al., 2010; Roman and Winter, 2004). The TGA results (Figures 10 and 11) confirm the evaporation in both untreated and cellulose treated BC samples which are

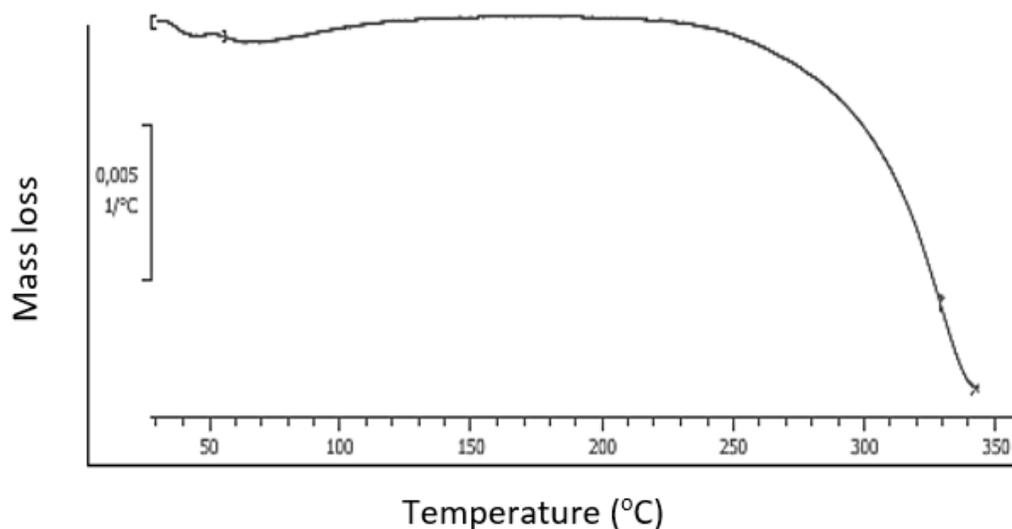


Figure 10. TGA thermogram of untreated BC sample.

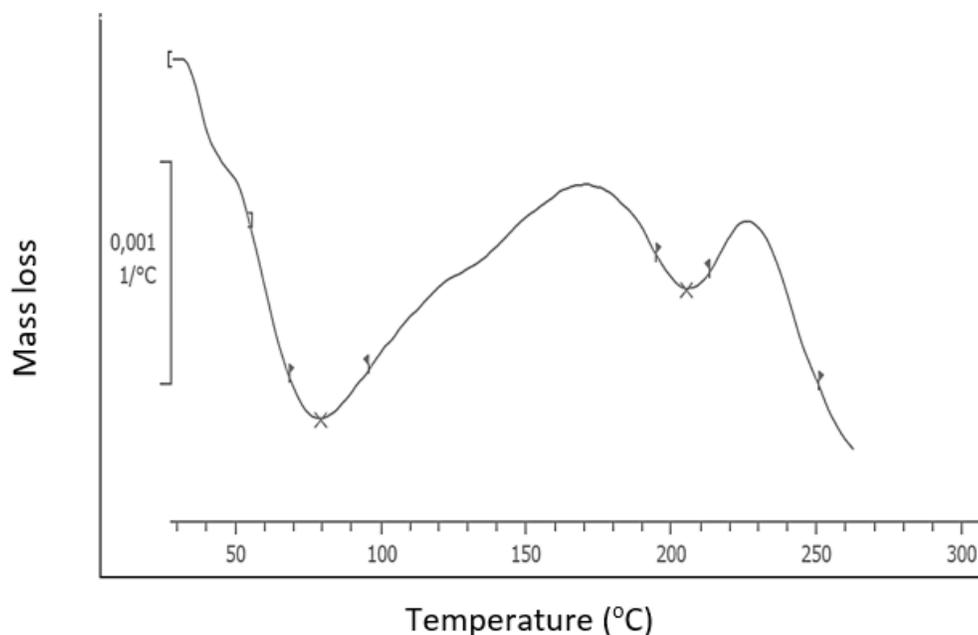


Figure 11. TGA thermogram of cellulase treated BC sample.

caused as a result of solvent or the presence of water in the BC samples. The probable presence of water could be as a result of sample exposure which thereby absorbs atmospheric moisture. The results from the TGA analysis also confirm the degradation of untreated BC after 200°C and that of treated BC after 150°C. The fast degradation of cellulase treated BC in DSC analysis was found to be consistent with the observed degradation in TGA analysis.

Conclusion

G. xylinus is considered to be the bacterial species with the most commercial interest (Andrade et al., 2010) and BC produced by *G. xylinus* could be of commercial interest and open new avenues in the field of degradable polymers. This study provides for the first time a fully detailed characterisation of pure bacterial cellulose before and after treatment with a commercially available

cellulase enzyme. This suggests an attractive prospect for the production and recycling of biopolymers from bacterial sources.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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