Properties of Bacterial Cellulose Produced in Grape Medium by Native Isolate *Gluconacetobacter* Sp

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ABSTRACT: During the production of grape wine, the occurrence of thick leathery pellicle at the air-liquid interface was found as a contaminant. The pellicle produced was investigated with a view to use as biodegradable polymer. The bacterium that is responsible for the pellicle production was isolated, characterized and identified as *Gluconacetobacter* sp. Pellicle was produced in pasteurized grape extract as well as in HS medium by the isolated organism in static conditions. The purified film was subjected for Fourier transform infrared spectroscopy and C^{13} solid NMR spectroscopy analysis, which confirmed the pellicle to be a cellulosic material. Scanning Electron Micrograph showed ultra fine network structure along with cells. The films were tested for its physicomechanical characters, barrier and thermal properties. The films of 25-μ thickness showed very high tensile strength (41.158 MPa) and elongation of 0.987 mm. The thermal properties of the films were characterized by Differential scanning calorimetry and Thermo gravimetric analysis. The melt peak temperature was found to be 111.65°C. The percentage of weight loss was found to be 20% at 327.86°C. Barrier properties (oxygen transmission rate and water vapor transmission rate), indicated a high oxygen barrier but low water barrier. This is the first report on the barrier properties of bacterial cellulose from *Gluconacetobacter* sp. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 120: 2835–2841, 2011

Key words: bacterial cellulose; Gluconacetobacter; water vapor transmission rate; oxygen transmission rate; thermal properties

INTRODUCTION

The production of cellulose is a characteristic feature of *Gluconacetobacter* sp. *Gluconacetobacter* species generally produce cellulose in liquid medium and form a floating pellicle comprising of cellulose, entrapped cells with other media ingredients. Bacterial cellulose (BC) is produced comparatively in larger quantities unlike other microbial polymers. It is considered one of the traditional foods among Philippine people and is also popular in other Asian countries, including Indonesia, Japan, and Taiwan, due to its distinctly soft texture and high fiber content.[1] BC displays many unique properties including high mechanical strength, highly crystalline, and an ultra fine highly pure nanofibril network structure with stability toward chemicals and high temperature.[2] In native state, BC has greater hydration, holding over a 100 times its own weight of water. These properties of biopolymer make it applicable in biomedical science such as temporary substitute for human skin in case of burns, ulcers, and other biotechnological fields.[3] It has been investigated as a potential scaffold for tissue engineering,[4] used as high quality speaker diaphragms and as food bulking agent.[5] BC production is also well known for its ease of production with cheaper source of raw material that is an added advantage.

The aim of this study was to characterize the BC produced from native isolate. Pellicle produced on pasteurized grape extract and Hestrin Schramm (HS) medium by the isolated organism in static condition. The bacterial polymer so produced is characterized for its structural and molecular properties by scanning electron microscopy, Fourier transform infrared spectroscopy (FT-IR), ^{13}C-nuclear magnetic resonance (NMR), physicomechanical, barrier properties, thermal characteristics of melt, decomposition by Differential scanning caloriemetry, and thermal stability by Thermo gravimetric analysis.

MATERIALS AND METHODS

Isolation and identification

The pellicle formed in contaminated grape wine was collected and was washed with sterile distilled water and cut into pieces (1 cm^2) under aseptic conditions.

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Cut pieces were placed in a sterile plastic bag containing Peptone saline solution. To obtain a diluted suspension, the contents were pressed manually to release the entrapped cells for 2 min.[6] The released cells in peptone water was serially diluted and streaked on to agar plates of HS medium and the plates were incubated at ambient temperature in inverted position for 48 to 72 h. Isolated colonies were picked and further purified. The purified colonies were inoculated into pasteurized grape extract and reconfirmed for its ability to produce pellicle. The isolate was identified as *Gluconacetobacter* species using standard microbiological methods.[7] Further confirmed by 16S rRNA sequencing,[8–10] and the sequence was deposited in GeneBank (National Center for Biotechnology Information Taxonomy, Bethesda, MD) with an accession no. *G. hansenii* UAC 09 (FJ655878).

The organism was maintained in HS slants at 4°C and sub culturing at regular intervals of two weeks.

Culture media

HS medium

Glucose 20 g/L, peptone 5 g/L, yeast extract 5 g/L, citric acid 1.15 g/L, Di sodium hydrogen phosphate 2.7 g/L, pH 4.5.[11]

Grape medium

Grape (Bangalore blue variety) was purchased from the local market, washed thoroughly with water and juice was extracted in a fruit juicer, collected in glass carbuoy. Cane sugar (5%) was added and the extract was pasteurized twice with an interval of 18 h. at 70°C for 15 min. Physical and Chemical parameters such as pH, total sugar,[12] reducing sugar,[13] total acidity, and total polyphenols[14,15] were tested for grape medium.

Inoculum preparation

HS medium was used for the production of inoculum. One loopful of culture was inoculated and grown on a rotary shaker (200 rpm) at ambient conditions for 24 h.

Production of pellicle

The pasteurized grape extract was inoculated with 5% inoculum (24 h grown in HS medium), incubated at ambient temperature in stationary condition for 2 weeks.

Purification of pellicle

Pellicle grown on the liquid surface was harvested once in a week in sterile condition. Washed thoroughly with water, immersed in 1*N* NaOH for 1 day at room temperature to remove the cells and other impurities embedded in the pellicle. The pellicle was rinsed thoroughly with water until a neutral pH was attained in the drained water. The pellicle was press dried in between the filter papers at 60°C till the film weight was maintained constant.[16–18]

Scanning electron microscopy

The sample preparation was carried out with 2.5% glutaraldehyde fixation overnight. Further, the sample was dehydrated in a graded series of ethanol from 10 to 100% and was then gold coated. The plated replica was examined for its Surface morphology using scanning electron microscope (LEO 435 VP Electron Microscope, Cambridge, UK).

Mechanical properties

Tensile strength (TS) and percent elongation (%E) at break of BC films from grape and HS medium were measured as per ASTM D 882/1995 using LLOYD's universal testing instrument (LLOYDS-50K, London, UK) instrument with initial grip separation of 50 mm and cross head speed of 100 mm/min. TS was calculated by dividing the maximum load for breaking the film by cross-sectional area and %E by dividing film elongation at rupture to initial gauge length at an ambient temperature (25 ± 2°C). Average of 10 measurements is reported.

Barrier properties

Barrier properties of BC were compared for films produced in grape and HS medium.

Water vapor transmission rate

Water vapor transmission rate (WVTR) of BC films were determined using the aluminum dishes as per ASTM E-96. About 50 sq mm diameter samples were sealed on a cup containing highly hygroscopic material like anhydrous $CaCl_2$. The film was placed on the cup and sealed all round using hot wax, leaving 50 sq mm surface area in circular form for the exposure. The cup with the wax was then rested for few minutes to reach room temperature. The prepared cup was weighed and placed in the humidity chamber maintained at 38°C and 90% RH gradient. An increase in weight due to absorption of moisture by $CaCl_2$, permeated through the film was measured hourly once. Weight gain graphs were plotted with respect to time, and linear least square method used

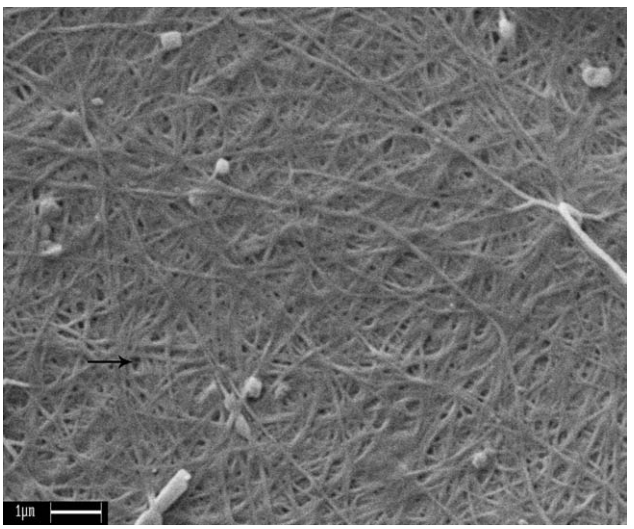

Figure 1 Sodium hydroxide (1*N*) treated BC film devoid of cells (pore size < 1 μm indicated by an arrow) at 30 K magnification.

to calculate WVTR as per the following equation and was expressed as $g/m^2/day$. Average reading of four samples is reported.

$$\text{WVTR} = \frac{\text{Slope(wt gain/day)}}{\text{Film area}(m^2)}$$

Gas transmission rate

Oxygen transmission rate (OTR) of BC films were determined volumetrically using permeability cell (Customs Scientific Instruments, NJ) as per ASTM D 1434 procedure. The test makes use of permeability cell consisting of two stainless steel discs that form cylindrical cavity when discs are superimposed. The film to be tested was clamped between the two discs using six equally spaced bolts after placing filter on the upper discs (as support) and a rubber gasket to ensure a pressure tight fit. The cell consists of a glass capillary in a vertical position to an opening in the center of the upper disc. Suitable gas inlet and vent lines were provided on both sides of the cells. Oxygen was supplied from surge tank at a constant pressure to the bottom of the cell. A short plug of mercury, contained in a capillary was displaced upwards by the permeating gas and this displacement gives the rate of permeation of the gas through the film material.

An electromechanical vibrator was used to avoid friction to the movement of the plug. The change in volume of permeates was measured as a function of time. The displacement of mercury versus time was plotted and slope of the straight line obtained. Gas transmission rate was calculated using the following formula and expressed as $cm^3/m^2/day/atmospheric$ pressure.

$$\text{OTR} = \frac{31620 \times \text{slope}}{\text{Pressure}}$$

Where 31620 is capillary constant. Average reading of four samples was reported.

Thermal properties

Differential scanning calorimetry

Various melt parameters of BC film were determined using differential scanning calorimeter (DSC Q200, TA instruments, Denwara USA). The experiment was carried out with sealed empty pan as the reference, with N_2 gas flushing. The sealed pan with sample (5–7 mg) was first cooled to $-50°C$, held isothermally for 1 min, and then ramped (10°C/min) to 400°C to obtain the thermograms.[2] Onset (T_o), crystalline melt temperature (T_p), and temperature of completion of the endotherm during melting and heat of enthalpy (ΔH) were obtained on thermograms using TA universal thermal analyzer 2000 software.

Themogravimetric analysis

A thermal weight change analysis instrument [thermogravimetric analysis (TGA) Q50, TA Instruments] was used to analyze the BC film. The TGA was employed to measure the amount and rate of change in weight of the material, either as a function of increasing temperature or time, in a controlled atmosphere. The sample (8–10mg) was kept in a platinum crucible and heated in the furnace, flushed

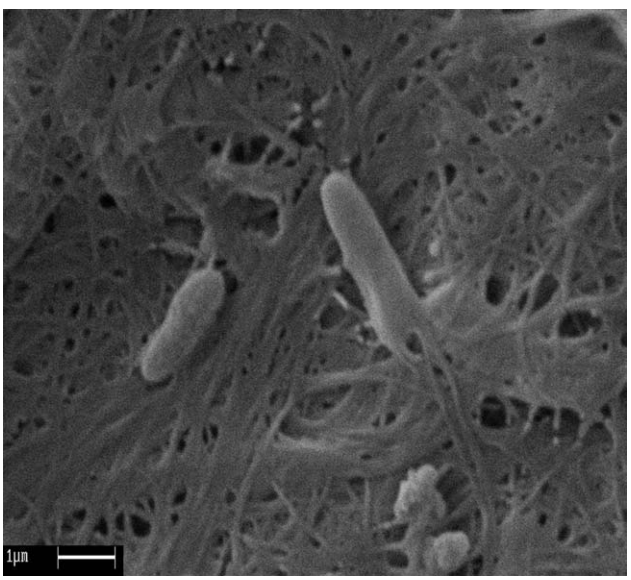

Figure 2 Scanning electron micrograph of BC with cells at 30 K magnification (rod shaped bacterial cells of 0.5–0.7 $\times$ 1.2–2.2 μm).

TABLE I
Physicomechanical and Barrier Properties of Bacterial Cellulose Film from Grape and HS Medium

Properties	Tensile strength (MPa/25μ)	% Elongation (mm/25μ)	WVTR ($g/m^2/day$)	OTR ($cm^3/m^2/day$/ atmospheric pressure)
BC from grape medium	41.158 ± 2.79	0.987 ± 0.07	2448.28 ± 6.12	415.27 ± 3.57
BC from HS medium	16.56 ± 1.28	1.675 ± 0.12	1453.21 ± 5.93	1962.67 ± 2.99

with N_2 gas at the rate of 40 mL/min, from 30 to 600°C, at the rate of 10°C/min.[2] The percentage weight loss and derivative weight loss were plotted against temperature. Result was analyzed using TA universal 2000 software.

FT-IR spectral studies

Thin films of 25 μm with uniform thickness were used for obtaining the IR spectra of BC films using FTIR-RAMAN Nicolet 5700. All measurements were carried out at 20°C in anhydrous conditions with air as the background. For each sample, 32 scans at a 2 cm^{-1} resolution were collected in the range of 4000–400 cm^{-1}.

Cross polarization-magic angle spinning (CP-MAS) ^{13}C-NMR analysis

^{13}C-NMR spectra of BC was recorded on a Bruker dsx300 spectrometer (Karlsruhe, Germany) utilizing cross polarization pulse sequence. The dry powder placed in a ceramic rotor was spun at the magic angle of 5 to 7.5 kHz, with the accumulation of $>$ 2000 scans at constant time of 2 min and a pulse (repetition) time of 5 min.

RESULTS AND DISCUSSION

Total sugar of the grape medium was found 17.6% and reducing sugar was 13.0%. The media had a pH of 2.89 with 0.1% acidity. The total polyphenol content was 0.14%.

Yield of the BC in grape extract was found to be 7.47 g/L as dry weight after 2 weeks of incubation under stationary condition at ambient temperature. Under the similar condition, in HS medium it was found to be 1.76 g/L. Earlier working with HS medium, a yield of 0.64 g/L. was reported by El-Saied et al.[19]

Scanning electron microscopy

The scanning electron micrograph of the press dried film revealed it to be a fine net work of less than 1 μm pores size (Fig. 1), as reported in earlier observations George et al.[20] The fibrillar nature was visible at 10 K magnification. NaOH treated film was devoid of cells, untreated film had cells with debris incorporated between fibrils (Fig. 2).

Physico-mechanical properties

Mechanical properties of films from grape and HS medium were compared in Table I. It was observed that the TS of BC films from grape was 41.16 MPa where as from HS medium was found to be 16.56 MPa. The increase of TS 148% in grape medium when compared with BC films of HS medium. The mechanical properties in comparison with synthetic polymer indicates that BC to be better than low-density polyethylene (LDPE),[21] which had values of 16.08 to 17.17 MPa.

It was also observed that the elongation percentage (%E) of BC films from grape medium was 0.987 mm when compared with BC film from HS medium which is about 1.675 mm (Table I). The %E of BC from HS medium was 69% more when compared with BC from grape medium. Earlier, Keshk[22] has noticed that impregnation of lignosulphonates increased the TS of BC. Increase was attributed to the increase in effective cross-sectional area that produces an increase in cross-sectional momentum. The other reasons may be due to the increase in the number of 1, 4 covalent bond.

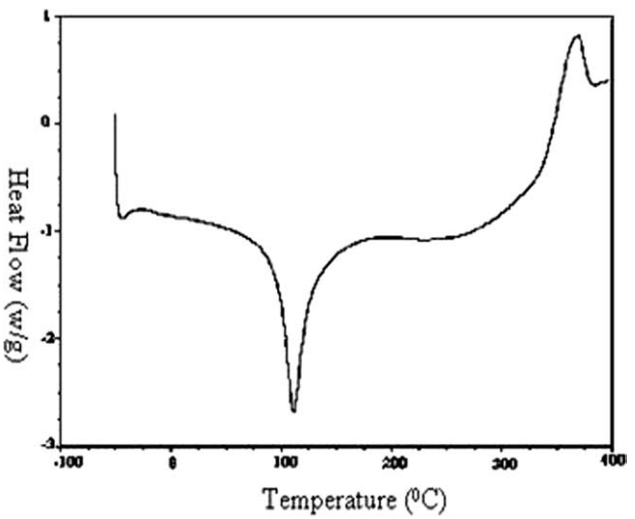

Figure 3 Differential scanning calorimetry thermogram of BC produced in grape medium.

TABLE II
Thermal Properties of Bacterial Cellulose Produced in Grape Medium. Data on Melt and Decomposition Characteristics of Bacterial Cellulose by Differential Scanning Calorimetry

	T_o (°C)	T_p (°C)	T_c (°C)	T_c–T_o (°C)	ΔH (J/g)
Endotherm	97.39	111.65	186.09	88.7	241.8
Exotherm	354.44	369.49	383.67	29.23	43.21

T_o, temperature onset; T_p temperature peak; T_c, temperature completion; ΔH, enthalpy of melt

Barrier properties

The transmission rate of a gas or a vapor in a polymer is a complex phenomenon and depend on various factors such as degree of crystallinity, crosslinking, chainscission, oxidative degradation, free radicle recombination, etc.[23] Values of WVTR and OTR of BC films are given in Table I. The values showed lesser (68%) WVTR in BC films from HS medium when compared with BC from Grape medium. The oxygen barrier property of BC films from grape medium showed 372% times better barrier to oxygen compared with BC from HS medium. Synthetic polymers normally have low WVTR (18–20 g/m^2/day) and a high OTR (8000–14000 cc/m^2/day).[24] Hence the present result is reverse of synthetic polymers.

Higher the degree of crystallinity lower is the permeability, because the crystalline regions are relatively impermeable compared with the amorphous area. For a given penetrant molecule, the rate of diffusion is governed by the nature of the polymeric chains and therefore changes in the molecular structure may influence its diffusional behavior.[23] Low-OTR value indicates low permeability of BC produced in grape medium (Table I). Because the crystalline regions are relatively impermeable, the present result indicates that the BC produced in grape medium has high crystalline structure when compared with BC from HS medium.

The present result indicates the influence of media on the properties of BC. Earlier, Raj et al.[24] have shown that incorporation of other components to LDPE changes the WVTR and OTR values. The change in barrier properties may be due to the salts and other constituents present in a natural grape media (undefined) compared to the defined HS medium. No such reports on BC are recorded as per the knowledge of the authors.

As the properties of film produced from grape medium was found to be better than the film from HS medium, further studies were carried out only on grape medium.

Thermal properties

DSC was used for analyzing glass transition temperature (Tg) and bulk crystallization kinetics for thermal characterization of macromolecules (melting temperature and decomposition temperature) (Fig. 3) The Tg of highly crystalline macromolecules like cellulose is difficult to detect properly using this technique because of the broad and flat heat flow curves for which step deviation from the baseline is comparatively less. To improve the resolution, relatively large samples (> 10 mg) is required.[2]

Heat flow curves of NaOH treated cellulose films produced from grape medium showed an endothermic peak at 111.65°C, which appeared to be the crystalline melting temperature of the polymer. At higher temperature at 369.49°C, an exothermic peak was observed, followed by the decomposition of cellulosic material. The calculated heat of crystallization (ΔH) was obtained by integrating the peak with time (Table II).

TGA is a continuous process, involving the measurement of sample weight in accordance with increasing temperature in the form of programmed heating. This method can be used to characterize any material that exhibits a weight change on heating and to detect the phase changes due to decomposition and oxidation. TGA provides better understanding of thermal decomposition behaviors and depends on several factors such as sample geometry, mass, compatibility, and heating rate.[2] The TGA curve is obtained by plotting percent weight loss

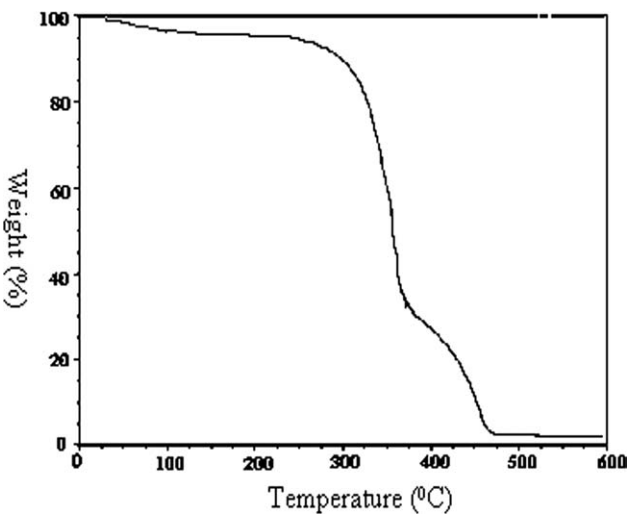

Figure 4 Thermo gravimetric analysis thermogram of BC produced in grape medium.

TABLE III
Thermal Properties of Bacterial Cellulose Produced in Grape Medium. Data on Themogravimetric Analysis of Bacterial Cellulose

Temperature at 10% weight loss (°C)	300.00
Temperature at 20% weight loss (°C)	327.86
Temperature at 98% weight loss (°C)	596.00
Decomposition temperature (T_{max}) (°C)	357.85

against temperature (Fig. 4). The initial loss below 200°C may due to dehydration, at higher temperature the cleavage of glycosidic bonds occurs and weight loss is rapid. The percent weight loss at 300°C was 10.3 and 98% weight loss was noticed at 596°C (Table III).

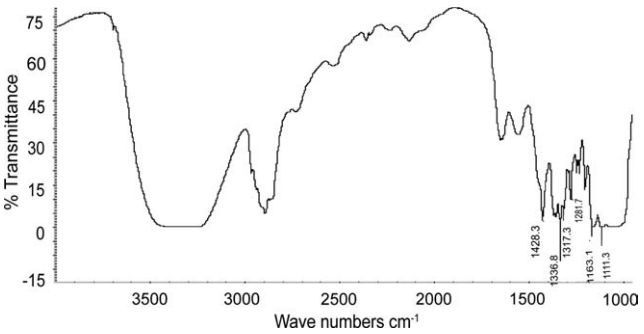

Figure 5 FT-IR spectrum of BC produced in grape medium.

Molecular characterization

FT-IR analysis of BC produced in grape medium (Fig. 5) indicates the most significant bands of cellulose and their corresponding assignments are akine to cellulose based on previous published references.[25–27] Cellulose is a biopolymer comprising of B-D-glucopyranose units linked together through β-1, 4 glycosidic linkages. A broad band observed between 3700 and 3000 cm^{-1} corresponds to the —OH stretching vibrations of cellulose and water molecules. The peak around 1640 cm^{-1} is due to the H-O-H bending vibration of absorbed water molecules in cellulose. The 1430 ($C—H_2$ bending), 1162 (C—O—C stretching), 1111 (Ring asymmetric stretching) and 895 cm^{-1} (Group C1 frequency) transmittance bands can be used to study the type of crystalline cellulose because, the crystalline cellulose I spectrum differs clearly in these bands from cellulose II. 1430 cm^{-1} band is a characteristic of crystalline cellulose I. If a cellulose fiber has a significant amount of cellulose II, this band moves towards 1420 cm^{-1} and the amount

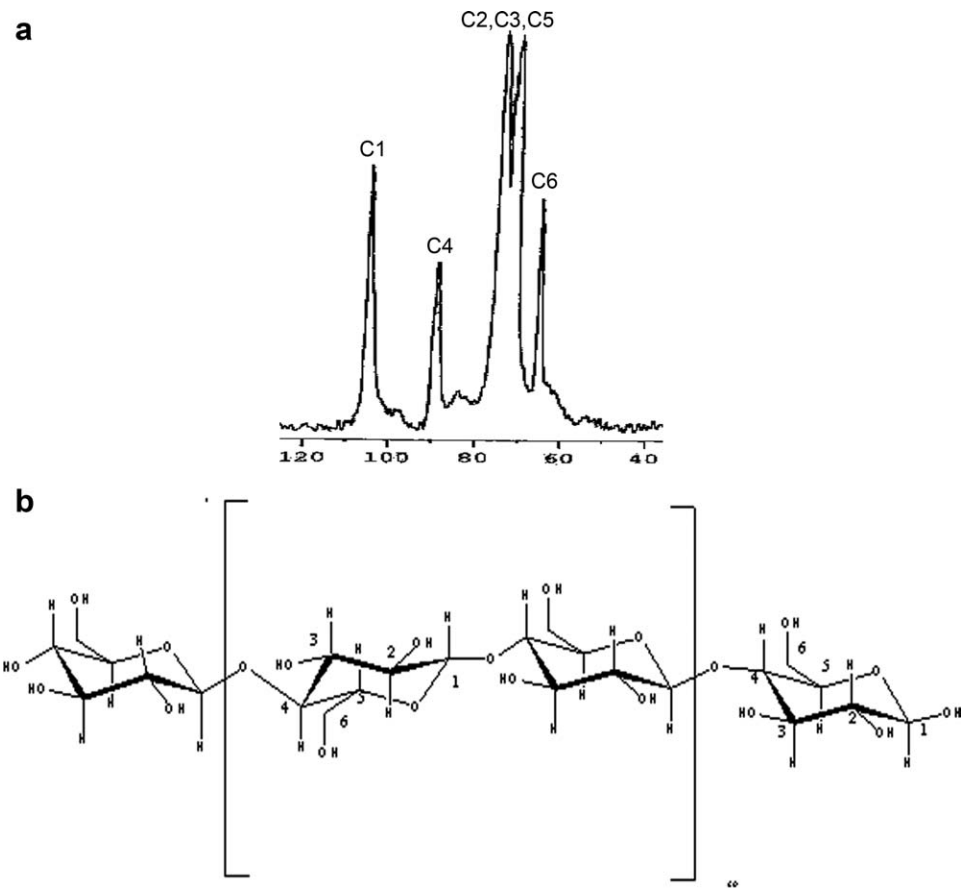

Figure 6 CP-MAS ^{13}C-NMR spectrum of BC produced in grape medium (A) and postulated structure (B).

of cellulose I decreases.[26] In the spectral region, 1162 cm^{-1} assigned to cellulose C-O-C bridges. In crystallized cellulose this band is located at 1163 cm^{-1}, whereas for amorphous cellulose it is located at 1156 cm^{-1}. The band at 895 cm^{-1} corresponds to crystalline cellulose I. The 1335-1316 doublet is assigned to the cellulose with high crystallized cellulose I content.[27] The frequency bands at 1375, 1335 (O—H plane bending), 1315 (CH2 wagging), and 1278 cm^{-1} (CH bending) indicated the presence of crystalline cellulose II.[25] In Figure 5, the characteristic bands at 1428, 1163, 1111, and 897 cm^{-1}(cellulose I) and 1336, 1317, and 1281 cm^{-1} (cellulose II) were found, which indicates a predominance of crystalline cellulose I, as in the mixture of crystalline cellulose I and II these bands also appear. Working with these results, the spectra of BC confirms that it is mainly composed of crystalline cellulose I with a negligible content of crystalline cellulose II.

The chemical nature of the BC was elucidated by ^{13}C solid state NMR. Figure 6 shows the ^{13}C solid state NMR spectrum of BC from grape indicating carbohydrate region as a large signal between 60 and 110 ppm. In the spectra of BC, only the regions of C1, C4, and C6 were subjected to the line-shape analysis during a considerable variation in chemical shift. The cluster of resonances between 70 and 80 ppm was attributed to the ring carbons C2, C3, and C5. In the spectrum of the BC the resonance regions associated with C4 and C6 include sharper signals (88.8 and 65.1). Multiplicity of signals in the spectra of crystalline cellulose was interpreted as arising from carbons in allomorphs of crystalline domains i.e., for C1 : 1α 108 ppm, 1β 104, and 106 ppm.[28] A tentative interpretation of the C4 region (1α 89.6 ppm, 1β 88.0 ppm) allowed us to draw some conclusions about the content of ordered forms of cellulose (intensity in the 86–92 ppm spectral region) and unordered (80–86 ppm) forms of cellulose. From the spectrum, C4 (88.8 ppm) and C1 (104.9) confirms ordered β form of cellulose. Similar pattern was observed by Nakai et al.,[29] indicating the structure of cellulose.

CONCLUSIONS

The results indicate that grape medium produced better quality BC than HS broth in terms of yield (almost five times more) and mechanical properties (TS exhibiting an increase of 148%). The TS being better than LDPE, (16.08 to 17.17 MPa) compared with for BC produced from Grape media (41.16 MPa). NMR and FT-IR data indicates the product to be cellulose with out any other groups. A high WVTR and low OTR, which is contrary to polymers, indicate its probable use in foods which requires low-OTR packing materials.

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