EFFECT OF ETHANOL AND MEDIUM ON BACTERIAL CELLULOSE (BC) PRODUCTION BY *GLUCONACETOBACTER XYLINUS* (PTCC 1734)

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The effect of medium and ethanol on bacterial cellulose (BC) production by *Gluconacetobacterxylinus* (PTCC 1734) under agitating culture conditions was studied. The results showed that the yield and characteristics of BC are influenced by different components of the media. BC produced in Hestrin-Schramm (H) and Zhou (Z) media had the highest dry-weight yield, which increased by the addition of 1% (v/v) ethanol. According to Field Emission Scanning Electron Microscopy (FE-SEM) and Energy-Dispersive Spectroscopy (EDS) analyses, BC fibrils grown in Z medium were more intertwined than those grown in H and Y media. The addition of ethanol resulted in BC with a dense structure, and high O/C ratios. XRD and FT-IR measurements showed a higher crystallinity for the BC grown in H medium, while the BC grown in Z medium containing ethanol contained more surface carboxylate groups. This study shows that the BC properties can be altered by the culture medium and by the addition of ethanol.

Keywords:bacterial cellulose, *Gluconacetobacterxylinus* PTCC 1734, FE-SEM, EDS, X-ray diffraction, FT-IR spectroscopy

INTRODUCTION

Cellulose, the most abundant terrestrial biomacromolecule, is predominantly produced by vascular plants.[1] It is composed of glucose monomers connected by β(1–4) glycosidic linkages to form long chains, with a degree of polymerization >5000. Because of the increased demand for natural cellulose and increased consumption of wood as a raw material of cellulose, deforestation is occurring worldwide and creating global environmental issues.[2] Therefore, an alternative source for cellulose production is necessary to compensate for a portion of wood consumption.[3]

Bacterial cellulose (BC) is an exopoly-saccharide produced from various species of bacteria, such as those of the genera *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, and *Salmonella*.[4]*G. xylinus* is an efficient bacterial species for producing BC at large scale. BC possesses special and unique properties that differ from plant-derived cellulose. It is chemically pure (free of other structural components, such as lignin and hemicelluloses), has a highly crystalline nano-structure, and a degree of polymerization that distinguishes it from other forms of cellulose.[5] When compared to plant-based cellulose, BC has a higher crystallinity, tensile strength, moldability, water-holding capacity (it can retain water up to 700 times its dry weight), biocompatibility, and transparency. Further, BC has a fiber network structure.[6-9]The ultra-high strength and toughness of BC make it an ideal raw material for use in high performance applications (composites, pharmaceuticals, food additives, textiles, mining, and refinery), where plant-derived cellulose is not suitable.[10]

BC can be produced in static, or agitated culture systems.[11]A static culture results in the

formation of a gelatinous pellicle of cellulose on the surface of the medium, while an agitated culture system produces pellets of irregularly shaped clusters, or star-shaped structures inside the medium.[12] Bacterial cellulose harvested from an agitated culture system generally has lower crystallinity, crystallite size, degree of polymerization, and I_α content than that produced from a static culture. However, agitated systems offer a low production cost, and production optimization possibility for an efficient and convenient production of BC.[13-15] In contrast, a static culture does not offer any optimization alternatives and the process demands more manual handling. Multiple studies have focused on optimizing BC production aiming at improving cellulose properties.[16-20]It was reported that the crystallinity of BC can be controlled by medium selection and culture conditions.[21] BC produced from a low quality date syrup feed showed a higher yield than that produced from sucrose.The effect of ethanol on BC production, by using isolated *Acetobacter sp.* A9 strain, was also studied.[22] It was reported that the addition of ethanol to the medium could lead to an increase in BC production. The use of ethanol in varying concentrationsin the BC culture medium has been studied and demonstrated to be promising for optimizing yield.[23-26]

To the authors' knowledge, the effect of ethanol and medium on BC production from *G. xylinus* PTCC 1734 has not been studied yet. Hence, the present study aimed at investigating the effect of ethanol addition and different types of medium on dry weight, pH, yield, sugar consumption, surface morphology, crystallinity, and chemical structure of BC produced from *G. xylinus* PTCC 1734 under agitated culture conditions.

EXPERIMENTAL

Stock culture

G. xylinus was obtained from the Persian Type Culture Collection (PTCC), strain number 1734. The strain was cultured on glucose yeast extract (GYE) agar containing D-glucose (100 g), yeast extract (10 g), peptone (5 g), CaCO3 (20 g), agar (25 g) per L.[27] After being cultured for three days at 28 °C, slants were ready to undergo preparations for pre-culture.

Pre-culture

Pre-culture medium (50 ml) composed of H medium[28]was placed in a 250 ml Erlenmeyer flask, and autoclaved at 121 °C for 15 min before inoculation. Then, the flask was cooled at room temperature, inoculated with the stock culture (a loop from a slant), and incubated in a shaker (150 rpm and 28 °C) for three days.

Culture conditions

After preparing the pre-culture, Hestrin-Schramm (H), Yamanaka (Y) and Zhou (Z) media (Table 1) containing glucose as carbon source, with (+) and without (-) ethanol, were cultured in 250 ml flasks containing 50 ml of medium. The media were inoculated at 10% (v/v) concentration, after being autoclaved at 121 °C for 15 min. The amount of ethanol was 1% (v/v), and the pH of all media was adjusted to 5.5 with NaOH. The culture media were incubated for 7 days at 28 °C under agitation (150 rpm).

Purification of cellulose

After the incubation period, the harvested BC was initially washed with 1% NaOH at 80°C for 1h, and then with distilled water repeatedly until a neutral pH was reached.

Table 1
Composition and concentrations of media

Composition	Media (%, w/v)		
	Hestrin-Schramm (H)	Yamanaka (Y)	Zhou (Z)
Glucose	2	5	4
Corn steep liquor (CSL)	-	-	2
Yeast extract	0.5	0.5	-
Peptone	0.5	-	-
Na_2HPO_4	0.27	-	-
Citric acid H_2O	0.115	-	-
$(NH_4)_2SO_4$	-	0.5	0.4
KH_2PO_4	-	0.3	0.2
$MgSO_4.7H_2O$	-	0.005	0.04

Determination of dry weight, pH and yield

BC production was recorded as dry weight of BC within the volume of medium (g/L). To determine the

dry weight of the cellulose sheets, they were oven dried at 45 °C for three days and then weighed. The pH of the remaining medium was measured after the cellulose sheets were harvested. The yield of the biosynthesis process was calculated as follows:

$$Yield\,(\%) = (m_o / C) \times 100 \quad (1)$$

where: m_o is dry weight of BC (g) and C is the weight of carbon source (g).

Sugar consumption

The amount of sugar consumption of the medium during the cultivation period (7 days) was measured using the DNS (dinitro-salicylic acid) method.[29] According to this method, the sugars chemically reduced DNS to 3-amino-5-nitrosalicylic acid in alkaline solution, and in this reaction, DNS underwent a color change from yellow to orange-red. Optical density (OD) was used to measure the absorbance by a UV/Vis spectrophotometer at a wavelength of 540 nm.

Field-emission scanning electron microscopy (FE-SEM) and energy-dispersive spectroscopy (EDS)

The surface morphology, shape and structure of BC microfibrils were analyzed by field emission scanning electron microscopy (FE-SEM, Hitachi SU 8090, 5 kV). Samples were mounted and gold-coated for preparation. The elemental composition of the samples was determined by energy-dispersive X-ray spectroscopy (EDS). Quantitative analyses were done, in triplicate, for both weight (wt%) and atomic (at%) percentages forcarbon (C), oxygen (O), nitrogen (N), and O/C ratio.

X-ray diffraction analysis (XRD)

X-ray diffraction patterns of BC (1 cm^2) were collected on an X'Pert pro MPD (multi-purpose diffractometer, Model PW3040/60) with CuK_α radiation generation at a temperature of 25°C, resolution of 0.001°, voltage of 40 kV and filament emission of 40 mA. Diffraction intensities were measured between 2θ of 5-50°. Crystallinity (Cr%) was calculated by the following equation:[30,31]

$$Cr(\%) = (S_c / S_t) \times 100 \quad (2)$$

where: S_c and $S_t$are the areas of the crystalline and total domains, respectively. Crystallite size (CrS) was estimated using the Scherrer equation:

$$CrS = K\lambda / \beta \cos\theta \quad (3)$$

where K is the shape factor (0.9), λ is the x-ray wavelength (1.54Å), β is the line broadening at half the maximum intensity (FWHM) in radians and θ is Bragg's angle.

Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared spectroscopy (FTIR) of dried BC was performed on a Bruker Equinox55 analyzer, equipped with a DTGS detector and a golden gate micro ATR. The spectra were collected at wave numbers ranging between 4000-600 $cm^{-1}$with an average of 16 scans. In addition, I_α and I_β contents were calculated using the peak heights at 750 and 710 cm^{-1}.[32]

RESULTS AND DISCUSSION

Effect of medium and ethanol on dry weight, yield and pH

The effect of medium type on dry weight, yield and pH was tested at 95% confidence interval. As can be observed in Fig. 1a, the dry weights of BCs produced in Z and H media were of 0.89 and 0.71 g/L, respectively (same group in Duncan's test), and were higher than that of BC produced in Y medium (0.39 g/L). These findings are in line with those obtained by Ruka*et al*.[21] H medium produced the highest yield of BC, whereas its carbon source was half to that of Z medium. It can beconcluded that BC production is influenced by the quantity and type of medium, as well as by its composition. Generally, the strain of bacteria, the source of carbohydrates, acidity, temperature, and culture methods are all important parameters in the production of BC by *G. xylinum*.[33] The pH in H medium decreased the least, i.e., this medium is more buffered when compared with the other two media. This finding supports the high BC yield in H medium. The decrease in pH is caused by the formation of gluconic acid, a by-product, in the media containing glucose.[34] Fig. 1b shows that the dry weight and yield of BC produced in the media containing ethanol were higher than those from media without ethanol. There was an increase of 57.7% and 54.9% in BC dry weight and yield, respectively.

The results clearly show that BC production is highly influenced by the addition of ethanol.[25,35] Ethanol can influence the enzymes involved in BC synthesis pathway. For example, when ATP (adenosine-5-triphosphate) is produced by ethanol, it inhibits the enzyme glucose-6-phosphate dehydrogenase. This enzyme shuffles glucose-6-phosphate into the pentose phosphate pathway. Therefore, an optimum amount of ethanol results in increasing BC production.[24,36] However, it should be noted that pH was not affected by ethanol addition.

Sugar consumption

The lowest and highest glucose consumption was observed in H and Y media, respectively (Table 2). H medium had the highest BC yield

and the most desirable composition to support production.

Field-emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy (EDS)

FE-SEM micrographs of BC produced under agitated conditions are shown in Fig. 2. All the micrographs show that the BC retained its network structure and twisting ribbons without any specific orientation. The width of BC fibrils produced in H medium was higher (41 nm) than those of the fibrils produced in Y (39 nm) and Z media (33 nm). The BC fibrils produced in Z medium appeared to be more intertwined than those from the other two media (Fig. 2 upper images). The addition of ethanol has significantly altered the morphology of BC (Fig. 2 lower images), showing a decrease in networking, and a dense flatter structure.

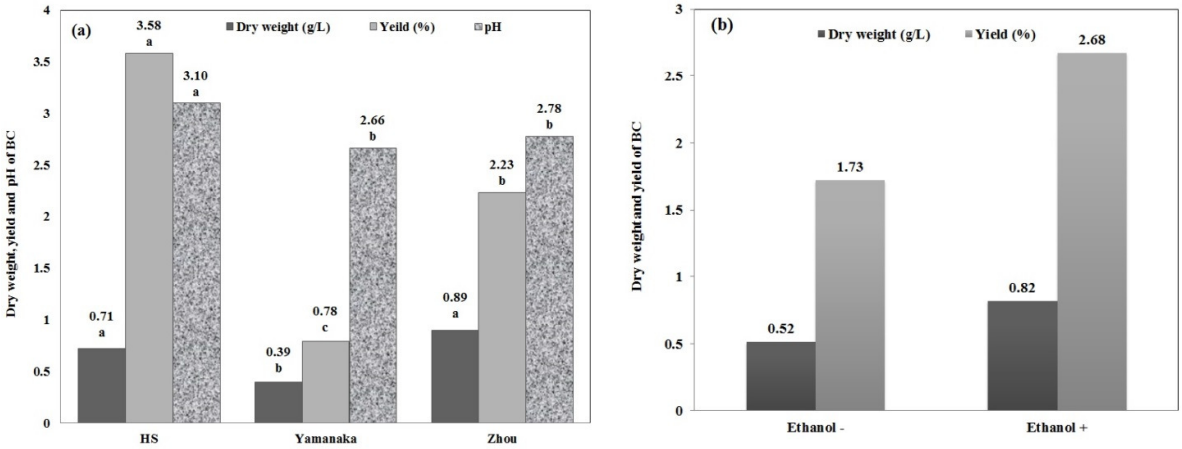

Figure 1: Effect of medium type and ethanol on dry weight, yield and pH of BC

Table 2
Initial andremnant glucose contents and glucose consumption of media

Medium	Initial glucose content (g/L)	Remnant glucose content (g/L)	Glucose consumption (g/L)
H-	20	2.36	17.64 (88.2)[a]
H+	20	2.18	17.82 (89.1)
Y-	50	2.01	47.99 (95.98)
Y+	50	2.33	47.67 (95.34)
Z-	40	2.61	37.39 (93.47)
Z+	40	3.07	36.93 (92.32)

a: percentage of sugar consumption

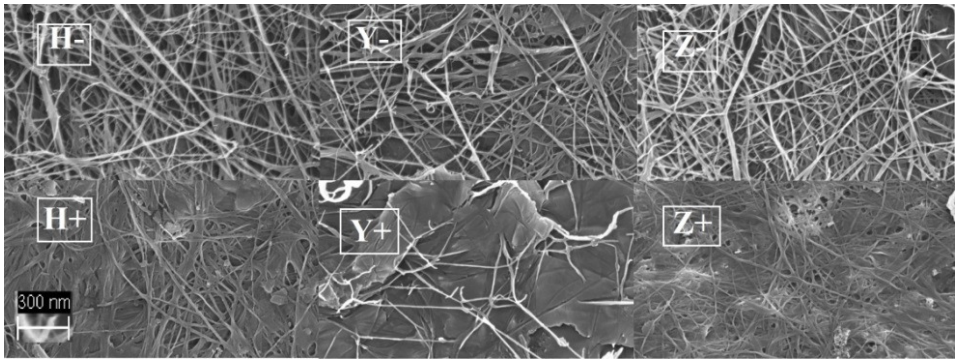

Figure2: FE-SEM micrographs of BC produced under agitation from different media (H, Y and Z) in the presence (+) or absence (-) of ethanol

Table 3
Surface chemical composition of BC

Medium	C (wt%)	N (wt%)	O (wt%)	O/C
H-	62.37 (68.16)[a]	3.96 (3.71)	33.51 (28.04)	0.54 (0.41)
H+	59.88 (66.37)	2.74 (2.60)	37.06 (30.84)	0.62 (0.46)
Y-	66.92 (72.26)	8.29 (7.69)	24.29 (19.77)	0.36 (0.27)
Y+	42.80 (49.88)	3.16 (3.17)	52.21 (45.87)	1.22 (0.92)
Z-	59.17 (65.80)	2.11 (2.01)	38.16 (31.86)	0.64 (0.48)
Z+	37.17 (44.15)	2.77 (2.82)	57.69 (51.57)	1.55 (1.17)

a: data included in parentheses are atomic percentages (at%) of elemental compositions

EDS analysis results for BC are given in Table 3. The O/C ratios of BC derived from the media with ethanol addition were higher than those of BC from the media without ethanol. This is confirmed by FTIR spectroscopy on BC – i.e. the O-H stretching band at 3345 cm^{-1}. The lower O/C ratio of BC from H+ medium (0.62), compared with those of BC from Z+ and Y+ media,is likely explained by its high crystallinity. The lowest BC O/C ratio was derived from medium Y- (0.36). This low O/C value was possibly caused by the purification process and medium-based organic impurities, such as N (weight 8.3%).

X-ray diffraction analysis (XRD)

XRD diffractograms of the BC samples are shown in Fig. 3. The main XRD peaks for all BC samples were located at 2θ = 14.7°, 16.2° and 22.4°, which correspond to cellulose I. No peaks were observed at 2θ = 12.1°, and 20.8°, which correspond to cellulose II.[31,33] Additional peaks were observed and may be assigned to components from the culture media. The Cr (%) of cellulose and CrS were calculated from the diffractograms. The results of detailed calculations are summarized in Table 4. BC from H and Y media had the highest and lowest Cr (%), respectively. It appears that the components from Y medium may interfere with the aggregation of BC microfibrils, resulting in a lower Cr (%).[21,37] The BC from Z medium had the lowest CrS size (6.5 nm); however, the BC crystallite size did not differ greatly among media.

Fourier transform infrared (FT-IR) spectroscopy

The structure of BC was determined by FTIR spectroscopy (Fig. 4). The main band assignments are given in Table 5. The bands observed between 3488 cm^{-1} and 3447 cm^{-1} are characteristic of intramolecular hydrogen bonded O-H stretching for cellulose.[38] The band intensity at 3345 cm^{-1} of BC harvested from medium H+ was more intense than those of BC from the other media types.

Table 4
Crystallinity (%), crystallite size (nm) and mass fraction of cellulose I_α and I_β

Medium	Crystallinity (%)	Crystallite size (nm)	Mass fraction of cellulose I_α	Mass fraction of cellulose I_β
H-	64.9	6.9	0.66	0.34
H+	76.2	6.9	0.48	0.52
Y-	53.9	6.7	0.68	0.32
Y+	53.2	6.9	0.71	0.29
Z-	55.9	6.8	0.71	0.29
Z+	60.6	6.5	0.60	0.40

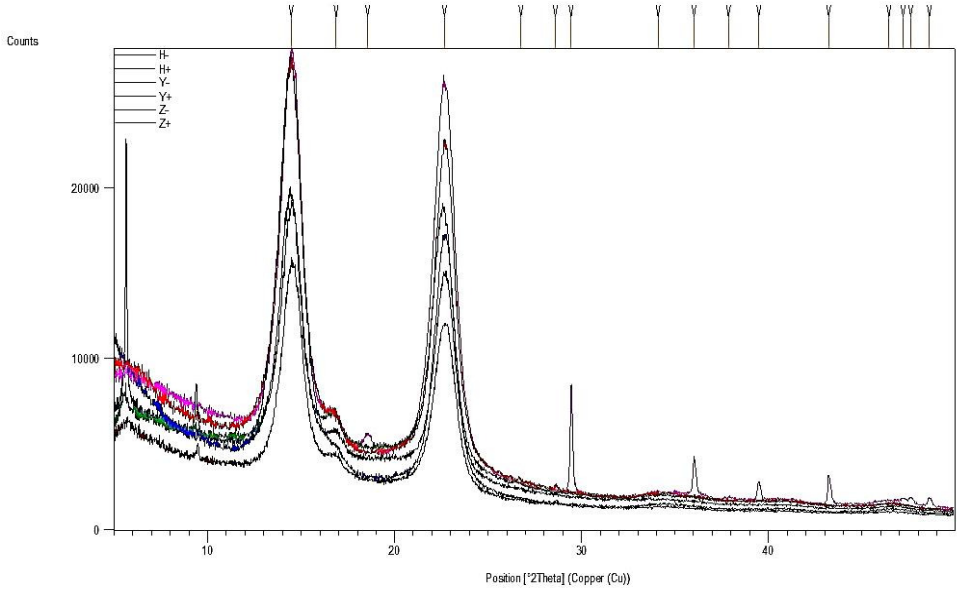

Figure 3: XRD of BCs

The hydrogen bond intensity (HBI) of cellulose is closely related to the crystallinity. The ratio of absorbance bands at 3400 and 1320 cm^{-1} indicates the cellulose HBI. This ratio for BC from medium H+ is around 1.09; while lower values were obtained for the other samples. The H+ medium BC had a highly ordered form, which resulted in strong hydrogen bonds. The ratio between the heights of the bands at 1372 cm^{-1} and 2900 cm^{-1} determines total crystalline index (TCI)of cellulose.[38] TCI data obtained by FTIR complete the crystallinity values obtained from XRD analysis. The highest and lowest TCI values of BC were from media H+ (1.04) and Y+ (0.84), respectively. The absorption band at 1644-1650 cm^{-1} in BC produced in medium Y+ was sharper than those of the others. This indicates that the moisture of BC was higher than that of the BC obtained under the other five conditions. Another difference in the spectra corresponds to the peak around 1403-1428 cm^{-1}; BC harvested from Z+ medium had a very sharp absorption band, corresponding to CH_2 symmetrical bending, or surface carboxylate groups.

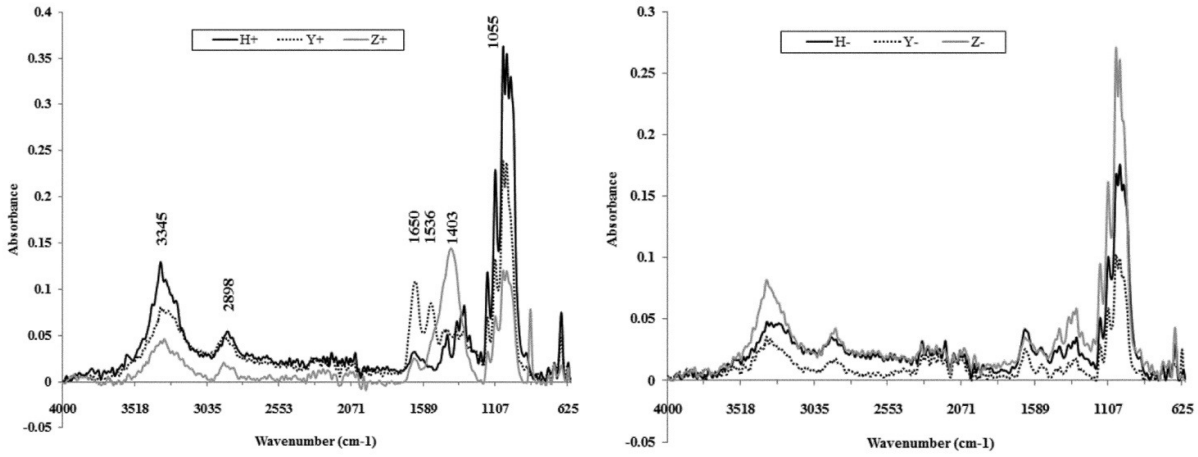

Figure 4: FTIR spectra of BC

Table 5
FTIR band assignments of BC[46]

Wave number (cm^1)	Assignment
Around 3345	OH stretching of cellulose I
Around 2898	CH_2 stretching
1644-1650	H-O-H bending vibration of absorbed water molecules
1543-1536	Protein amide II absorption
Around 1428	CH_2 symmetrical bending or surface carboxylate groups
1314	CH_2 wagging
1146-1160	Anti-symmetric bridge COC stretching
1107	C-O bond stretching
1050-1055	Ether COC functionalities and C-OH stretching vibration
870-900	Out of plane CH bending vibrations
665-670	Out of plane C-OH bending

The absorption bands near 750 and 710 cm^{-1} are assigned to cellulose I_α and I_β, respectively. The ratio of cellulose I_α and $I_\beta$forms in BC are given in Table 4. The highest crystallinity value was for BC produced in medium H+ (76.2%), while its cellulose I_α component was the lowest. The decrease in cellulose I_α showed the enhanced crystallization of cellulose I_β; which is presumably attributedto ethanoladdition. Furthermore, mediumconstituents and moisture content of BC can affect the aggregation of microfibrils and influence its crystallization. BCs harvested from media Y+ and Z- had similar absorption assigned to –OH groups (3345 cm^{-1}). However, absorbed water and surface carboxylate groups of BC produced in Y medium were more pronounced than those of BC from medium Z-; which caused a decrease in crystallinity. There is a strong correlation between crystallite size and cellulose I_α contents. BC obtained from medium H+ was an exception from this rule.BC from medium Z+ had a crystallite size of 6.5 nm and the lowest proportion of cellulose I_α (0.6).

CONCLUSION

BC was successfully produced from *G. xylinus* culture. BC production was highly influenced by the culture medium (H, Y and Z), the final product showing a networked intertwined structure. The addition of ethanol increased BC production in all medium types. However, the obtained BC was shown to have a dense flat structure with thinner fibrils and an oxygen-rich surface.

Cellulose crystallinity analysis (FTIR and XRD) showed that BC from H and Y media had the highest and the lowest crystallinity, respectively. Cellulose I_α structure in BC was decreased in H+ medium, which presumably enhanced the crystallization of cellulose I_β and possibly influenced by the addition of ethanol.

The thus-produced BC can be used as nano-cellulosic fibers for applications in biocomposite materials.

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