

# SPENT BLACK LIQUOR AS AN ALTERNATIVE CARBON SOURCE FOR THE SYNTHESIS OF BACTERIAL CELLULOSE

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Bacterial cellulose is identical in chemical composition to cellulose extracted from lignocellulosic biomass, but with partial difference in structural characteristics. These differences, specifically its purity, make it valuable, but its production processes are quite expensive. In the present work, spent black liquor resulting from cotton pulping, as a major industrial waste stream, was investigated as an alternative carbon source in the production of bacterial cellulose (BC) using *Acetobacter xylinum*. XRD results of the produced cellulose showed that the crystallinity of the BC was lower than that of cotton pulp alpha-cellulose. SEM evaluation confirmed the nano-size of the produced cellulose, while its structure was evidenced by FT-IR analysis. The effect of altering the culture media on some structural features of the produced BC was thoroughly discussed and it was suggested that the spent liquor could be added in amounts of up to 25% for BC production in standard cultures (HS).

**Keywords:** *Acetobacter xylinum*, bacterial cellulose, Hestrin-Schramm culture, spent liquor

## INTRODUCTION

Bacterial cellulose (BC) production from *Acetobacter xylinum* was first patented in 1886 by A. J. Brown.<sup>1</sup> He observed that cells of *Acetobacter* produced cellulose in the presence of glucose and oxygen in a static culture. These bacteria use glucose or other types of sugars and glycerol or other organic materials as carbon source, to produce cellulose chains through the bioprocess of fermentation. The culture is first filled with a slimy material and shortly followed by cellulose fibre formation.<sup>2</sup> Later, electron microscopes proved the nano-sized fibrillar structure of bacteria-derived cellulose.

BC has significant advantages compared to plant cellulose, whose fibrils are oriented randomly and contain more amorphous areas. It has been reported that a never-dried membrane of bacterial cellulose is nearly pure, containing 99.1 wt% of water, of which 0.3 wt% is bound and 98.8 wt% is free water. The surface area of bacterial cellulose is 200 times higher than that of cellulose isolated from softwoods, with a tensile strength similar to that of steel.<sup>3</sup>

Early experiments demonstrated the remarkable mechanical properties of bacterial cellulose sheets, with Young's modulus higher than 15 GPa. The mechanical properties of thin membranes are slightly affected by the fermentation conditions, as well as by the sheet preparation procedure, including pressing and drying. Structural studies have attributed the high Young's modulus to the unique super-molecular structure, in which fibrils of biological origin are preserved and tied tightly by hydrogen bonds.<sup>4</sup> A sheet-shaped material produced from bacterial cellulose has remarkable mechanical properties, and has been proven useful for reinforcing paper produced from conventional pulp.<sup>4</sup>

Bacterial cellulose has many advantages over plant cellulose: it is pure, being devoid of the lignin and hemicelluloses of plant cellulose. Also, the isolation of BC is facile and it does not require bleaching. Its other unique properties include high crystallinity degree, water retention value, tensile strength and flexibility.<sup>5</sup> However, bacterial cellulose is traditionally produced from costly cultures, containing glucose as carbon source and other nutrient resources, and thus its production is relatively expensive, which restricts its application in value-added products. In line with the trend towards a sustainable use of industrial wastes to help solve crucial global economic, environmental and energy issues, utilizing carbon and nutrient materials from inexpensive sources, such as agricultural and forestry residues, is an exciting approach to diminishing the production costs of bacterial cellulose. In this respect, a number of industrial residues, such as sugarcane molasses,<sup>6</sup> sugar beet molasses,<sup>7,8</sup> corn steep liquor,<sup>9-11</sup> sulphite pulping black liquor,<sup>12</sup> and fruit extracts including coconut,<sup>13</sup> pineapple,<sup>14</sup>

grape, apple, orange, pear<sup>15</sup> etc., have been studied as potential carbon sources for BC production. The utilization of these wastes for BC production will also help in waste management, reducing the cost of waste disposal for industries.<sup>16</sup>

At present, the soda pulping process is widely used in Iranian pulp mills. The process produces waste liquor, called “black liquor”. Black liquor is a significant water pollution source, and much research has been focused on overcoming this issue over the years, by investigating its reuse, recovery, sustainable utilization, chemicals extraction etc.<sup>17-22</sup> In spite of all efforts, black liquor as waste is currently abandoned and discharged as effluent, or is concentrated to burn in recovery boilers, generating energy, and, possibly, to recycle inorganic chemicals required for the papermaking process.

Compared to other categories of black liquors, alkaline cotton pulping black liquor has some particular properties, including intensive dark colour, more dissolved organic contaminants (*i.e.*, cellulose, oligosaccharides, and fatty alcohol), a higher pH and lower lignin content. The lower lignin content results in a relatively low heating value and makes conventional alkali recycling ineffective for the treatment of cotton pulp black liquor. On the other hand, the discharge of black liquor, without any treatment, causes severe environmental pollution. Therefore, finding an effective way to utilize cotton pulping black liquor is necessary for cotton pulp mills.<sup>19</sup> Besides, it deserves more valorization for fabrication of higher value-added products than its current uses.<sup>23</sup>

Considering the importance of utilizing economical materials and industrial residues as potential nutrients in bacterial cellulose production, the present research studied the spent liquor of alpha-cellulose production as a potential nutrient. This work aims to use cotton pulping black liquor generated at Parchin Chemical Industries as a potential nutrient for *Acetobacter xylinum* bacterium growth, leading to BC production. It is suggested that the obtained BC can be further utilized by this company in cosmetics and hygienic products.

## **EXPERIMENTAL**

### **Bacterium preparation and preservation**

*Gluconacetobacter xylinus* strain PTCC No. 1734 was purchased in lyophilized form from the Persian Type Culture Collection (PTCC) Center of the Iranian Research Organization for Science and Technology (IROST), and used for the present study. To activate the mentioned microorganism, normal saline was used. An agar culture in Petri dishes was used for the activation of the bacterium. The prepared cultures were kept at 0-4 °C and were used as required. BC was produced both in Schramm-Hestrin (HS) medium and spent liquor obtained from Parchin alpha-cellulose plant, separately, and in specified proportional combinations, and the products were compared with each other.

### **BC production in HS**

#### ***Agar plate culture medium preparation***

The agar plate medium was prepared by dissolving 100 g/L glucose, 10 g/L yeast extract, 20 g/L calcium carbonate and 15 g/L agar in an Erlenmeyer flask sealed with cotton and foil (according to the protocol of PTCC), and sterilized at 121 °C for 15 minutes in an autoclave. Then, the culture medium in the Erlenmeyer flask was transferred to Petri dishes under laminar air flux in a microbiological hood, where 1 to 2 mm thick films were formed, and kept for 24 h to facilitate the formation of solid culture medium. The agar plates were sealed by parafilm and stored in a refrigerator (0-4 °C) to be used as required.<sup>12</sup>

#### ***Bacterium culturing and propagation***

Four points were inoculated on each Petri dish, containing culturing substrate, using a metal micro-loop under laminar air flux in a microbiological hood. The Petri dishes were sealed by parafilm and were incubated at 28 ±2 °C for 24 hours (according to the suggested protocol of PTCC).

#### ***Hestrin-Schramm culture medium preparation for BC cultivation***

An amount of 20 g/L glucose, 5 g/L Bactopeptone, 5 g/L yeast extract, 2.7 g/L potassium phosphate and 1.15 g/L citric acid were dissolved in 100 mL of distilled water in a 250 mL Erlenmeyer flask. The pH of the inoculation culture medium was 5.5. The flasks were sealed with cotton and foil, and were sterilized in an autoclave at 121 °C for 15 minutes.<sup>12</sup>

The prepared HS culture medium was cooled in laminar air flux in a microbiological hood. The activated bacteria on the Petri dishes were transferred to flasks containing HS, using a micro-loop. The flasks were again sealed by cotton and foil, and were incubated at 30 °C. After 2 to 3 weeks, a thin membrane of cellulose was formed at the surface of the culture medium.<sup>3</sup>

### Cellulose production in spent liquor

The liquor utilized in the present study was generated from the cotton linter cooking process at Parchin Chemical Industries, and contains NaOH, lignin, extractives and pentosans.

### Spent black liquor (SBL) preparation

The pH of the spent liquor was 12. The preparation of the liquor consisted in pH adjustment with  $\text{NH}_4\text{OH}$ , followed by aeration with compressed air,<sup>26</sup> according to TAPPI standard of T222 om-02. Since the pH required for bacterial growth is 5-6, 70% sulphuric acid was added to the liquor until pH 5 was reached, with lignin precipitation. Lignin deposits were filtered on a Buchner funnel, and the filtrate was utilized for bacterial cellulose production. The spent black liquor (SBL) replaced the glucose contained in the HS solution, as a potential nutrient, in varied percentages: 10, 25, 50 and 100% (Table 1). In the 100% SBL addition ratio, glucose was thoroughly removed from the HS substrate.

### Preparation of bacterial cellulose membranes

After formation of the bacterial cellulose membrane, the pH of the culture medium was decreased to 4.5. The culture medium substrate was removed from the flask and a certain volume of 0.5M NaOH was added to the flask to submerge the membrane, followed by heating in a water bath to 90 °C (it should be noted that the alkali is used for inhibiting the growth of bacterium). This procedure was carried out in three replicates, to remove the bacterium cells from the membrane, after which the membrane was washed with distilled water at ambient temperature<sup>12,24</sup> (Fig. 1).

Cellulose membranes were placed on glass Petri dishes and were dried at 105 °C for 2 hours.<sup>12</sup> The membrane dried completely under the specified drying conditions and therefore adhered to the glass, which made it necessary to use a sharp tool to separate it – this led to rupturing of the film.

Table 1  
Bacterial culture media used in the study

Notation	Description of culture medium
HS	Standard Hestrin-Schramm culture medium
90HS+10LQ	90% standard culture medium + 10% liquor
75HS+25LQ	75% standard culture medium +25% liquor
50HS+50LQ	50% standard culture medium + 50% liquor
25HS75LQ	25% standard culture medium +75% liquor
100LQ	100% liquor

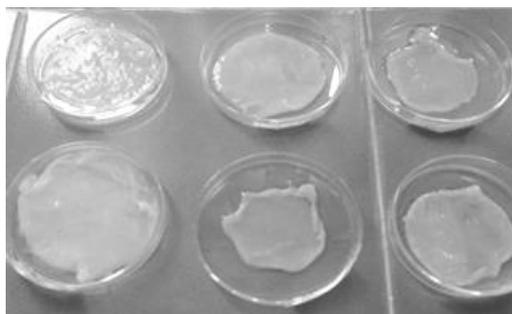


Figure 1: Bacterial cellulose membranes after washing with NaOH and distilled water

### Characterisation of bacterial cellulose

The bacterial cellulose yield was calculated by using Equation 1:<sup>25</sup>

$$\text{Cellulose yield (\%)} = \frac{\text{Dry Weight of the produced cellulose (g)}}{\text{Weight of primary glucose (g)}} \times 100 \quad (1)$$

The viscosity and degree of polymerization (DP) of the cellulose were determined according to SCAN standard CM 15:88.

For FTIR analysis of the bacterial cellulose, 4 mg of the cellulose was mixed with potassium bromide in a ratio of 1 to 100, and the prepared KBr tablets were studied by a Bruker Tensor27 FT-IR spectrometer. The absorbency of the samples was determined with 4  $\text{cm}^{-1}$  resolution and 16 scans were performed for each sample. The range of frequency for all experiments was 400-4000  $\text{cm}^{-1}$ .

In order to study the crystalline structure of the bacterial cellulose, a Philips X-Ray Diffraction device was employed. The X-ray powder diffraction data of the samples were collected at ambient temperature over the 2 $\theta$  range of 10-110°.

X-ray diffraction allows studying the crystallinity degree of particles. To determine the crystallinity degree, Segal's method defined in Equation 2 was employed. The crystallinity degree is the ratio of crystalline cellulose to the whole material, including both crystalline and amorphous areas:<sup>27</sup>

$$X_{CR} = \frac{I_{200} - I_{AM}}{I_{200}} \times 100 \quad (2)$$

where  $I_{200}$  is related to the peak with the highest intensity and  $I_{AM}$  is related to the peak at  $2\theta=18$ .

For SEM analysis of the bacterial cellulose, a KYKY-EM3200 Scanning Electron Microscope was used in this study.

## RESULTS AND DISCUSSION

### Production yield of bacterial cellulose

According to Figure 2 and based on Duncan's test results, the HS standard culture showed the highest cellulose yield, and replacing the glucose contained in the HS substrate with an increasing amount of spent black liquor caused a decreasing effect on the production yield of bacterial cellulose. As may be noticed in the figure, there is no significant difference in the bacterial cellulose yield between the HS standard culture and 90HS+10LQ, on the one hand, and 90HS+10LQ and 75HS+25LQ, on the other. This decrease can be attributed to the substitution of the carbon source in the HS culture with the liquor. It should be noted that, during cooking of cotton linter, a considerable quantity of hemicelluloses contained in raw linter is separated and released into the black liquor. These hemicelluloses existing in linter are mainly pentosanes and are converted to pentose sugars throughout decomposition in soda pulping.<sup>28,29</sup> It seems that the distinction between the sugar contained in the liquor (pentose) and the glucose in the HS standard culturing medium has been the reason for the reduction in the yield.<sup>30</sup> However, indirect biosynthetic pathways of BC, where glucose is first transformed into metabolic intermediates, such as pentose analogues, which are then incorporated into BC via uridine diphosphate glucose (UDP-Glucose), make pentoses a potential alternative carbon source, besides glucose.<sup>31</sup> Figure 2 also reveals that increasing the amount of liquor over 25% in the HS medium led to halting the bacterial growth, with no cellulose production. It is assumed that low molecular weight hydroxyl acids, resulting from cellulose and hemicelluloses degradation in soda pulping, as well as extractives<sup>12</sup> and some aromatic organics in black liquor could certainly inhibit the microbial growth and therefore the BC production.<sup>22</sup>

### Effect of altering the culture medium on bacterial cellulose degree of polymerization

The degree of polymerization (DP) of cellulose produced in HS and HS+LQ (in different ratios) is shown in Figure 3. The data illustrated in the figure suggest that the bacterial cellulose produced in HS standard medium has significantly higher DP, compared to the cellulose produced in HS+LQ.

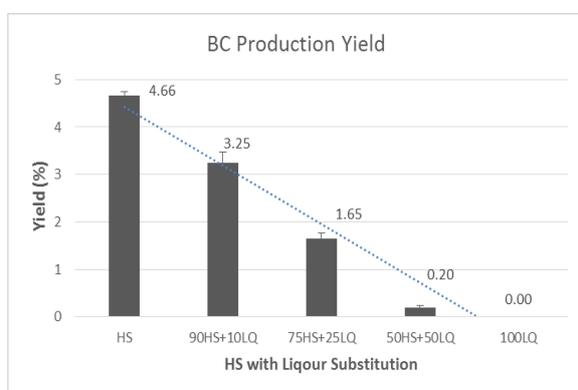


Figure 2: Cellulose production yield in different investigated media

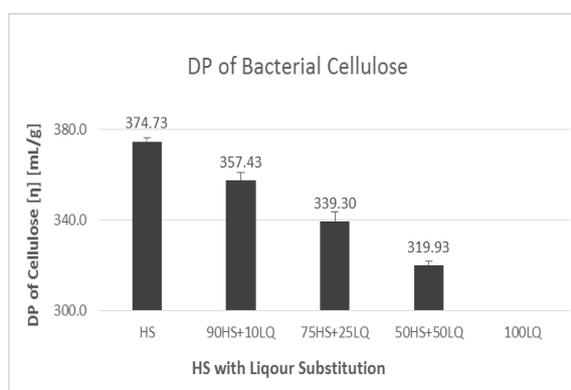


Figure 3: DP of bacterial cellulose produced in different investigated media

As Figure 3 implies, the DP of the cellulose produced in the culture media containing liquor is lower than that of cellulose produced in standard HS culture medium, specifically, the use of liquor instead of glucose resulted in 11% decrease in DP. This loss was attributed to the decrease in the nutrient amount (glucose) required for bacterial growth, even though the sugar present in the alpha-cellulose production liquor is pentose, which is considered a suitable substitute for glucose. Also, it

has been previously reported that the DP of BC could be affected by many factors, such as medium, fermentation mode and carbon sources.<sup>32</sup>

### FTIR analysis of bacterial cellulose

The FTIR spectra of bacterial cellulose produced in HS standard culture medium and altered medium with the addition of black liquor are presented in Figure 4 (75HS+25LQ was selected for further analyses as the optimum medium with black liquor addition). The absorption spectrum peaks at 1013 and 1021  $\text{cm}^{-1}$  in the spectra corresponding to HS and 75HS+25LQ culture media are indicative of glycosidic bonds (C-O-C) in the cellulose chains of bacterial celluloses grown in both media. The peaks at 1372 and 1374  $\text{cm}^{-1}$  in both spectra suggest the C-H and C-OH bonds, respectively, in the produced cellulose. Also, for both media, the peaks at 1648 and 1632  $\text{cm}^{-1}$  indicate the H-O-H bond, which is attributed to water absorbed by cellulose. Besides, the peaks at 701, 761 and 848  $\text{cm}^{-1}$  in the cellulose produced in HS medium, and the peaks at 2852 and 2921  $\text{cm}^{-1}$  in the cellulose produced in 75HS+25LQ medium are associated with C-H and  $\text{CH}_2$  bonds. The peaks at 2852 and 2921  $\text{cm}^{-1}$  are also associated with hydroxyl groups and are found in the spectra corresponding to both standard HS and liquor containing media.<sup>10,33-35,25,36</sup>

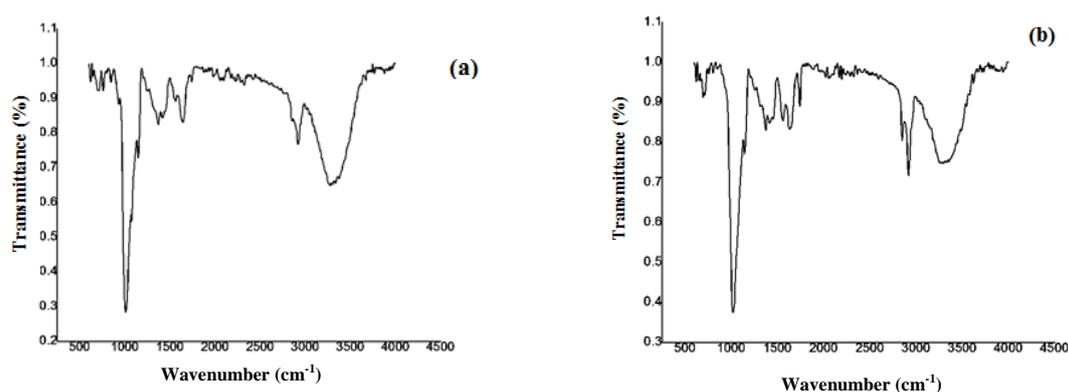


Figure 4: FTIR spectra of bacterial cellulose produced in (a) HS standard medium, and (b) 75HS+25LQ medium

### XRD analysis of bacterial cellulose

XRD analysis of the BC produced in HS culture medium revealed that the highest intensity attributed to the angle of  $2\theta = 19.92$  was 286, while at  $2\theta = 18$  the intensity was recorded as 166 (Fig. 5a). Based on these data and Segal's equation, the crystallinity of the BC grown in HS culture was calculated as 41.96%.

According to Figure 5b, the highest intensity peak in the BC produced in 75HS+25LQ culture was 299, which was assigned to  $2\theta = 19.64$ , while the intensity at  $2\theta = 18.2$  was 193. Using Segal's equation, the crystallinity of this BC was calculated as 35.45%.

In the literature, the values reported for the crystallinity of pulp fibers produced from cotton linters were very diverse, varying between 50% and 96%.<sup>10,27,37-38</sup> Excluding the effects of several factors, including the origin of the cotton, pulping and processing sequences or the measurement procedures, it is assumed that the existence of crystalline dimorphism of cellulose and the discovery of two families of native cellulose can explain the number of inconsistencies in the crystallographic study of cellulose in the last fifty years.<sup>39</sup> The crystallinity of the BC studied in the present work was inferior to that of cotton. Also, when glucose was substituted by black liquor in the culture medium, there was a 7% crystallinity reduction.

Many factors can affect the crystallinity of BC, including the cultivation method, carbon sources, pH, agitation speed, temperature, fermentation time and drying methods.<sup>40</sup> In previous studies, the crystallinity of BCs was reported to be as low as 46.7%, when the carbon source was sucrose.<sup>41</sup> Also, the results of yield and DP attested this loss as normal, emphasizing the importance of carbon source and the effect of the shift from glucose to pentose.<sup>42</sup>

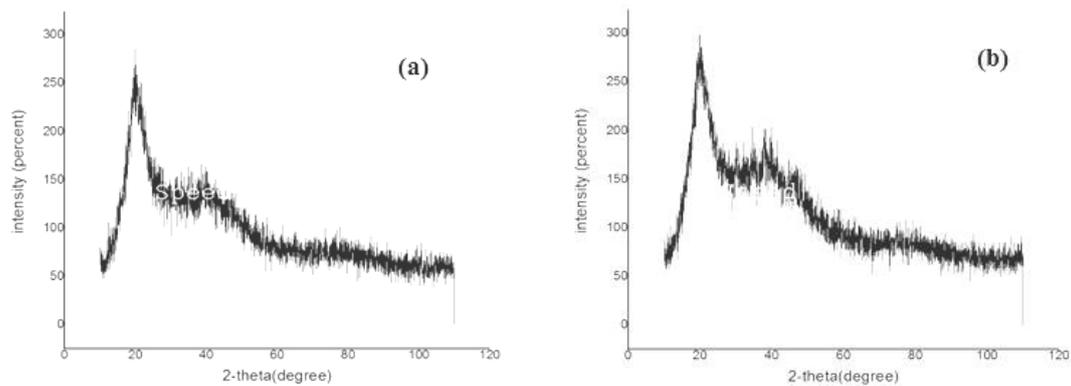


Figure 5: XRD patterns of BC produced in (a) HS culture medium and (b) 75HS+25LQ medium

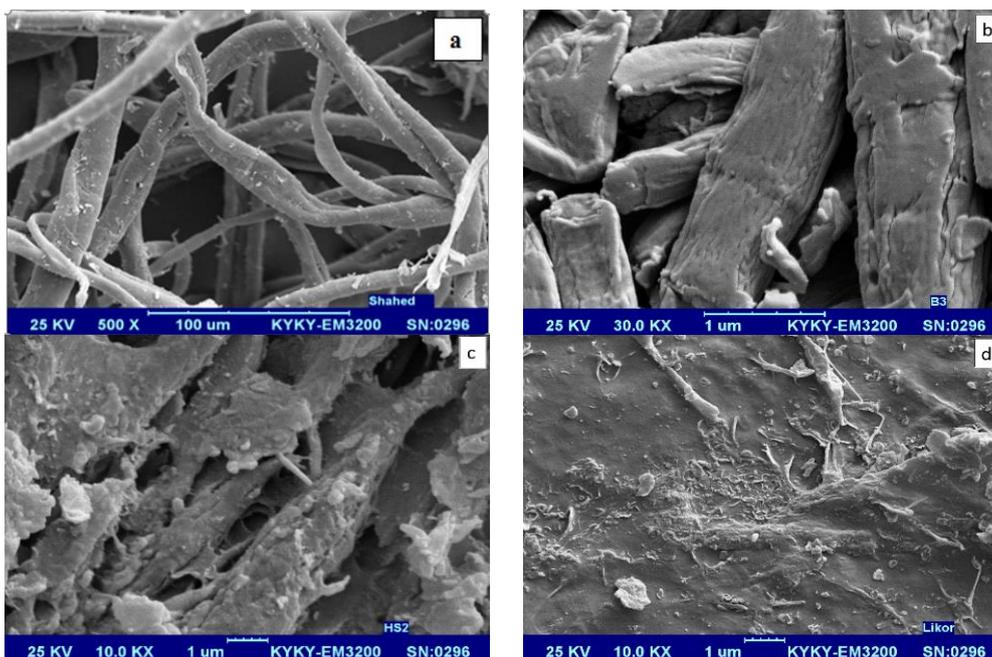


Figure 6: SEM images of (a) alpha-cellulose (for comparison purposes), and BC produced in (b) 90HS+10LQ medium, (c) 75HS+25LQ medium and (d) 10HS+90LQ medium

### SEM analysis

SEM images of BC produced in HS+LQ culture confirmed the reticulated structure of BC, containing fine and pure cellulose fibers, characterized by random arrangement. The strings were coiled and curved, resulting in a reticulated and dense structure (Fig. 6b). The micrographs reveal fibril bundles with the width within the range of 35-67 nm. The length and width of the BC fibers were observed to be lower than those of cotton alpha-cellulose fibers shown for comparison purposes in Figure 6a. The results obtained are in agreement with those reported previously in the literature, in terms of both length<sup>32</sup> and width.<sup>43</sup>

### CONCLUSION

The present study investigated the use of cotton pulping black liquor as a carbon source for bacterial cellulose production. The results indicated that a maximum 25% substitution of glucose in HS standard medium with black liquor is feasible, while higher percentages of black liquor hinder the growth of bacteria or even limit it to zero. The DP and crystallinity of the produced BC displayed lower values, compared to those of alpha-cellulose pulp, and the increasing proportion of liquor as a replacement for glucose in the growth media brought about 11% and 7% final reduction in DP and crystallinity, respectively. SEM analysis established the nano-sized structure of BC and FT-IR spectroscopy evidenced the cellulosic composition of the obtained product. The low yield production of BC reveals the need for an enrichment of the culture medium with more nutrients, possibly using

trace elements and corn steep liquor (CSL), particularly in the case of partial addition of black liquor as carbon source. It is expected that such an approach would ameliorate the structural features of the produced bacterial cellulose. Finally, it is concluded that the black liquor generated by Parchin Chemical Industries Co. could potentially be utilized as carbon source in HS standard media, by partially replacing the glucose. The valorisation of this important waste in this way would solve the problem of its disposal, avoiding its discharge into the environment and water pollution.

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