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Bacterial Cellulose Cultivations Containing Gelatin Form Tunable, Highly Ordered, Laminae Structures with Fourfold Enhanced Productivity

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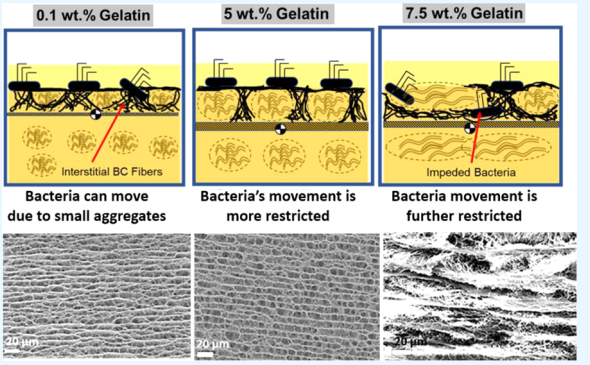
ABSTRACT: Manipulation of bacterial cellulose (BC) morphology is important to tune BC properties to meet specific application requirements. In this study, gelatin was added to cultivation media at 0.1−7.5 wt %. After cultivations, gelatin was removed from the BC matrix, and its effects on BC matrix characteristics and fermentation production efficiency were determined. Higher contents of gelatin in cultivation media (up to 5%) resulted in BC that, from scanning electron microscopy observations, had larger pore sizes and formation of a lamina morphology that was highly unidirectional. Crystallinity remained unchanged between 0.1 and 5 wt % gelatin concentrations (92−95%); however, it decreased to 86% at a gelatin concentration of 7.5 wt %. Mechanical properties showed a positive trend as both the specific modulus and specific strength values increased as the gelatin concentration increased to 5 wt %. A breakdown in the ordered structure of the BC matrix occurs at 7.5 wt % gelatin, with corresponding decreases in the specific modulus and specific strength of the BC. The productivity increased by almost 4-fold relative to the control, reaching 1.64 $g{\cdot}L^{-1}h^{-1}$ at the 2.5 wt % gelatin content. Also, the water holding capacity increased by 3-fold relative to the control, reaching 306.6 g of water per g BC at the 5.0 wt % gelatin content. The changes observed in these BC metrics can be explained based on literature findings associated with the formation of gelatin aggregates in the cultivation media and an increase in gel stiffness seen at higher media gelatin concentrations. Overall, this work provides a roadmap for manipulating BC properties while creating highly organized lamina morphologies.



INTRODUCTION

Bacterial cellulose (BC) has desirable characteristics such as bio-compatibility, low electrical conductivity, low density, and high mechanical strength.[1−8] These make BC useful for a diverse set of industrial applications such as high-quality paper, textiles, composite membranes, foods (nata de coco), artificial skin, blood vessel grafts, binding agent for fibers, loud-speaker diaphragms, cosmetics, and more. To date, Nata de coco is the main commercial product of BC, which is harvested from static fermentation by using coconut water as the nutrient.[9] Expansion in the use of BC will require low-cost carbon sources with concurrent increases in fermentation process parameters (e.g., carbon source utilization, volumetric yield, productivity) and performance metrics that are tuned to application requirements.[5,10−17] Overcoming these constraints will provide green BC matrices with tailored porosity, density, physical properties, and other conducive attributes for a wide range of application needs.

Komagataeibacter xylinus, a Gram-negative aerobic bacterium, secretes cellulose from cellulose synthetic sites located at pores within membranes. There are generally 50−80 of these synthetic sites that reside in the bacterial membrane. Due to the higher oxygen tension at the air−water interface, these aerobic bacteria strictly grow at the air−water interface. Hence, BC is formed at the top of cultivations, while previously formed layers move down below the surface forming the BC pellicle. Extruded cellulose molecules assemble into single elementary nanofibers with a diameter size of ∼1.5 nm due to hydrogen bonding to form elementary nanofibers that further assemble into ribbon-like nanofibers that have a width of about 50 nm. Ribbons, which consist of about 1000 individual glucan chains, organize in various ways depending on the culture conditions. General features are the organization of ribbons

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into dense layers, interstitial fibers connecting dense layers, and a distribution of pores.[10−17]

Particularly relevant to this work is the fact that additives to cultivation media may enhance the mechanical and chemical properties of the mesoporous network matrix as well as improve the fermentation efficiency.[18−20] Examples include observed increase in productivity and/or pore size by the addition of polyethylene glycol (PEG), potato starch, agar, and carboxymethyl cellulose (CMC) to cultivation media.[21−30] For example, BC cultivations with gelatinized potato starch granules increased the pore size from a reported 3−4 μm without potato starch to 40 μm in static cultures containing 4 wt % potato starch.[22] This large shift in the pore size was solely attributed to the addition of the potato starch and its subsequent swelling and solubilization of starch components during a preheating gelatinization process. In another example, concentrations of 0.1−1 wt % agar added to BC cultivations increased the volumetric yield of BC by up to 2-fold (5.5 g/L to 11.6 g/L) depending on the production strain.[27] Additionally, Cheng et al.[30] reported that, during cultivations in flasks, *Acetobacter xylinum* produced 8.2 g/L BC by inclusion of 1% carboxymethyl cellulose (CMC) in BC growth cultivations. However, instead of large BC masses, small pellets were formed. Also, CMC caused a small decrease in BC crystallinity that the authors attributed to the attachment of CMC to cellulose chains during nanofibril formation.

Gelatin is the partially hydrolyzed form of collagen, with an average molecular weight of 10,000 to 300,000 g/mol. Furthermore, it is a common waste product for industrial food manufacturing processes.[31,32] Gelatin forms chains in a coil conformation. However, during cooling in aqueous solutions, these chains undergo a coil-to-helix transition, thus aligning them within a semi-solid matrix.[33−35] At higher wt % concentrations (>1 wt %), a three-dimensional network forms during cooling that consists of associations between helical chains.[33,36] Given the effects described above of viscous additives, as well as the ordered gelatin structures formed that vary as a function of concentration, gelatin-containing media provide an interesting milieu for BC formation.

Few studies have been conducted to investigate the nature of gelatin as a direct additive to BC growth. Taokaew et al.[37] and Chen et al.[38] prepared BC-gelatin composites where gelatin was integrated into the matrix. Taokaew et al.[37] reported BC-gelatin composites with gelatin concentrations from 1 to 10 wt %. They identified that maximum BC productivity occurs at 3 wt %. Also, incorporation of gelatin in the matrix led to optically transparent matrices. In contrast, Chen et al.[38] found that the ideal gelatin concentration for productivity and desired mechanical properties is 0.5 wt %. Both studies crosslinked the BC-gelatin matrices and described properties of the resulting composite gels. For example, crosslinking gelatin within BC composites reduces the re-swelling ability while significantly enhancing the matrix strength.[38] In other reports, gelatin and BC in a segmented form were used to form crosslinked composites with sufficiently large pore sizes for tissue engineering.[39−42]

This study focused on gelatin-free BC matrices where, after cultivations with gelatin in the BC formation media, the gelatin was removed and replaced with distilled water. Motivation for this work was to ascertain, over a wide range of gelatin concentrations (0.1−7.5 wt/v), whether the viscoelastic culture medium, formed as a result of gelatin addition, could lead to changes in BC formation efficiency as well as matrix morphology parameters while also benchmarking against a culture devoid of gelatin. The work herein reveals important changes in culture volumetric yield, productivity, and carbon source utilization as a function of gelatin in cultivations. Furthermore, we show how prominent shifts in BC mat morphology including density, porosity, crystallinity, fibril organization, and orientation can be achieved by systematically changing the media gelatin content. Cross-sectional scanning electron microscopy (SEM) provided values of average pore size, fiber diameter as well as information associated with BC layer thickness, spacing, and directionality. Macroscopic properties such as matrix water holding capacity and mechanical properties are also reported. Overall, the results herein demonstrate that the presence and concentration of gelatin in cultivation media provide a powerful tool to manipulate BC matrix parameters.

MATERIALS AND METHODS

Bacterial Cellulose Preparation. The method used follows that described in the literature.[43] In summary, a seed culture of *Komagataeibacter xylinus* ATCC 700178 was prepared by inoculating 2 mL of a bacterial glycerol stock in 50 mL of Hestrin−Schramm (HS) medium and performing the cultivation in 250 mL Erlenmeyer flasks for 5 days at 30 °C. The Hestrin−Schramm (HS) medium consisting of 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L sodium phosphate dibasic, 1.05 g/L citric acid, and 40 g/L mannitol was purchased from Millipore Sigma. It was prepared in a 1 L media storage bottle and pH balanced with 3 M sodium hydroxide to 5.0. The autoclave-sterilized HS-mannitol medium was transferred using sterile 25 mL serological pipettes into sterile Petri dishes (10 cm diameter). After 27 mL of HS-mannitol medium was added, it was inoculated with 3 mL of seed culture (10% v/v).

The protocol for the control cultures (without gelatin) is identical to that of static cultivations where gelatin was included within cultivation media. The inoculum, 10% (v/v), was transferred from seed cultures to the Petri dishes, and cultivations were performed statically for 168 h at 30 °C. All media components, including gelatin (0, 0.1, 2.5, 5, and 7.5 wt %), were autoclave-sterilized and comprised the initial formulation of cultivation media (30 mL).

Post-processing of Cultivations. Removal of cells, gelatin, and HS broth from BC mats was performed following literature protocols.[44] In summary, BC mats were washed in 600 mL of a 3% sodium hydroxide (NaOH) solution at 80 °C for 3 h in an 800 mL beaker, with magnetic stirring, to remove cell debris and gelatin. If clear mats were not obtained, they were incubated overnight in a fresh 3% sodium hydroxide solution with magnetic stirring at room temperature to further remove non-BC substances. After BC mats appear transparent, further cleaning of the mats was performed at a constant flow of distilled water overnight. Selected mats were then lyophilized for water removal.

Measurements and Characterizations. The method used for scanning electron microscopy (SEM) analyses followed a literature protocol.[43] Mat cross-sectional images were recorded on a Supra 55 scanning electron microscope at 5−8 kV. Sectioning of BC mats was performed by first securing them to a scored glass slide and then fractured to achieve a live edge. The resulting cross sections were sputter coated, and SEM images were analyzed using ImageJ software.[45] ImageJ analysis gave values of BC matrix pore diameter, distance

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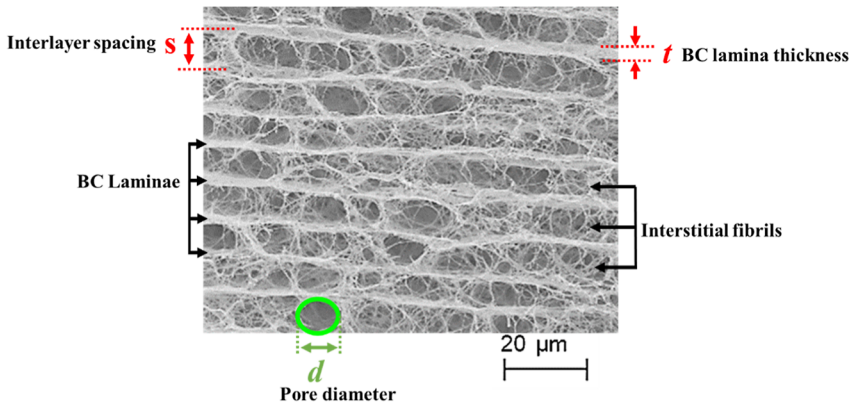

Figure 1. Representative SEM cross-sectional image and morphological features of interest in BC samples synthesized in gelatin media.

between dense BC nanofiber layers (laminae), and the interstitial fiber density. This data resulted from measurements of several representative images for each mat cross section. Detailed descriptions regarding the determination of laminae spacing/thicknesses and fiber densities between these layers are presented below (see Figure 1). The interlayer spacing was measured from the top edge of a fiber dense layer to the bottom edge of the next dense layer.

Wide-angle X-ray scattering (WAXS) was used to measure percent crystallinity with samples scanned from 10 to 40° of 2θ at 40 kV and 40 mA. Calculation of %-crystallinity (CI) was performed by a previously published method[46] by using eq 1

$$\mathrm{CI} = \frac{I_{002} - I_{\mathrm{AM}}}{I_{002}} \times 100\% \quad (1)$$

where I_{002} is the intensity of the peak at 2θ of approximately 22.5° and I_{AM} is the intensity at the peak of 2θ at approximately 18°. The product is multiplied by 100 to provide a crystallinity percentage.

The wet and dry weights were determined gravimetrically using a Sartorius analytical balance. Dry thickness was measured using a Mitutoyo digital APB-2D micrometer at 6 points within the BC mat. The cellulose productivity (P) of each BC pellicle was determined using eq 2

$$P = \frac{M_{\mathrm{BC\ dry}}}{V_{\mathrm{nutrient\ broth}} \times t_{\mathrm{incubation}}} \quad (2)$$

where $M_{\mathrm{BC\ dry}}$ is the mass of the dry pellicle measured after lyophilization, $V_{\mathrm{nutrient\ broth}}$ is the constant volume (30 mL) of the culture medium, and $t_{\mathrm{incubation}}$ is the total time of incubation (168 h). The carbon conversion efficiency (CCE) was determined using eq 3

$$\mathrm{CCE} = \frac{M_{\mathrm{BC\ dry}} \times C_{\mathrm{BC}}}{M_{\mathrm{Mannitol}} \times C_{\mathrm{Mannitol}}} 100\% \quad (3)$$

where C_{BC} and C_{mannitol} are the carbon fraction of the BC and mannitol dry weight, respectively, and M_{Mannitol} is the mannitol mass supplied to the culture. The water content (WC) of the BC pellicles was calculated based on eq 4

$$\mathrm{WC} = \frac{W_{\mathrm{w}} - W_{\mathrm{D}}}{W_{\mathrm{D}}} \times 100\% \quad (4)$$

where W_{W} is the mass of the wet pellicle and W_{D} is the mass of the dry pellicle after lyophilization. Measurements were conducted on a minimum of three replicates for each sample type.

Mechanical properties of the BC films were determined by tensile testing of five rectangular (20 mm long) hydrated samples with a gauge length of 20 mm and a width of approximately 5 mm (hydrated samples). The films were cut using a tungsten carbide roller cutter such that five rectangular samples were obtained from each BC pellicle. The samples were prepared and tested following the same protocol as outlined elsewhere.[43] A piezoelectric load cell (Kistler 9256C1, Switzerland), with a sampling frequency of 100 Hz, was used to collect the force data during this process.

In addition to BC tensile testing, the gelatin media used for BC synthesis were also mechanically characterized using a macro-indentation method inspired by the Bloom Test.[47] Cylindrical samples were prepared by casting 30 mL of a nutrient media solution containing different gelatin concentrations in the Petri dishes used for BC culture. The sample diameter was fixed at 100 mm, and the sample height was 5 mm for all concentrations of gelatin. Indentation of the samples was carried out using a cylindrical platen of diameter 36 mm. The platen was affixed to a linear motion stage, and indentation was performed at a constant rate of 1 mm/min. A piezoelectric load cell (Kistler 9256C1, Switzerland), with a sampling frequency of 100 Hz, was used to collect the load data during the indentation test. To compare the relative stiffness of the gelatin media, the load value at a fixed indentation depth of 1 mm was extracted from the indentation load versus depth curve. Due to the limited sensitivity of the load cell, all gelatin samples were characterized except for the 0.1 wt % sample that had the lowest stiffness. Three replicate samples were used for each experiment.

Infrared spectra were recorded using a Perkin-Elmer spectrometer by ATR-IR between 500 and 4000 cm^{-1}. Sixteen scans at a resolution of 1 cm^{-1} were evaluated and referenced against air. Samples were prepared by first lyophilization and then converting them to powders with a mortar and pestle.

RESULTS

Physical Appearance of Films. Figure 1S displays photographs of BC films prepared with 0% and 5% gelatin in culture media. They are similar in appearance and feel upon handling. While it appears that the BC 0% gelatin film is more translucent than the film prepared with 5% gelatin, the irregular surface features and film thickness variations between

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Table 1. Production Metrics and Material Properties of BC. The Values Listed Are the Mean and Standard Deviation from at Least Three Cultivations Conducted under Identical Conditions

culture medium	control (0% Gelatin)	0.1 wt % gelatin	2.5 wt % gelatin	5 wt % gelatin	7.5 wt % gelatin
productivity ($g{\cdot}L^{-1}h^{-1}$)	0.42 (±0.077)	0.77 (±0.051)	1.64 (±0.092)	1.29 (±0.043)	0.56 (±0.039)
%-carbon conversion efficiency (CCE)	16.9 (±1.42)	17.8 (±1.02)	27.1 (±1.86)	21.3 (±0.88)	9.3 (±0.79)
density (g/cm^3)	0.206 (±0.007)	0.16 (±0.009)	0.027 (±0.001)	0.014 (±0.0002)	0.046 (±0.001)
crystallinity (%)	93.4 (±1.4)	92.5 (±0.9)	92.2 (±1.1)	94.6 (±1.2)	85.6 (±0.7)
water content (w/w of water relative to BC)	99.2 (±4.81)	141.3 (±4.82)	298.2 (±3.45)	306.6 (±1.25)	177.5 (±2.87)
dry weight (g)	0.182 (±0.016)	0.153 (±0.012)	0.326 (±0.022)	0.256 (±0.011)	0.111 (±0.009)
dry thickness (μm)	112.9 (±5.95)	123.9 (±5.81)	1522.7 (±27.7)	2251.1 (±41.2)	311.6 (±23.2)

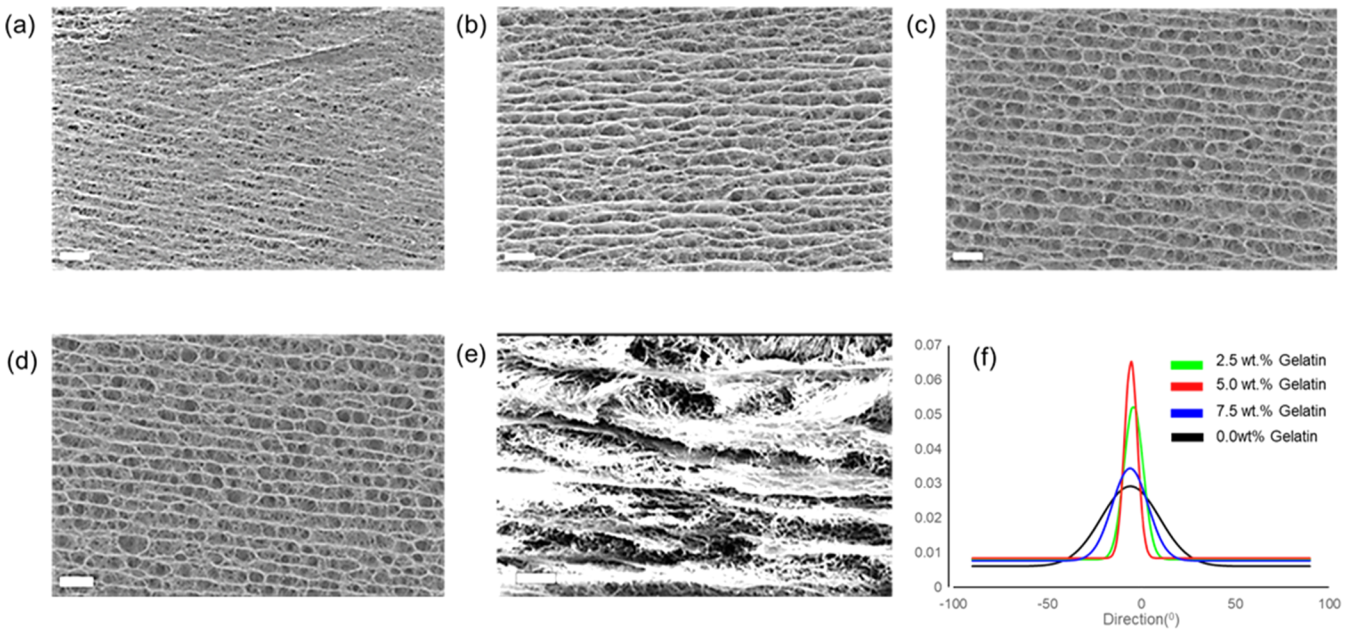

Figure 2. Characteristic meso-scale (2.5kx) SEM images of BC cross sections (a), control −0% gelatin (b), 0.1 wt % gelatin (c), 2.5 wt % gelatin (d), 5 wt % gelatin (e), 7.5% gelatin bacterial cellulose pellicles (scale bar = 20 μm) (f), directionality histograms of representative SEM images (a−e).

these films diminish the value of interpretations of these differences in appearance.

Gelatin Removal from Films. To determine whether the post-processing protocol successfully removed gelatin from BC matrices, FTIR spectra of gelatin, BC prepared without gelatin, and BC prepared with 5% gelatin in the medium were recorded (Figure 2S). Gelatin shows amide 1 and 2 bands centered at 1629 and 1525 cm^{-1}, respectively, in addition to other unique vibrational bands. These bands are not present in the FTIR spectrum of BC films prepared with 5% gelatin. Furthermore, the FTIR spectra of BC prepared with 0 and 5% gelatin in culture media are nearly identical. Hence, from FTIR, there is no evidence of residual gelatin remaining in BC films after the post-processing protocol (see above).

Relative Stiffness Measures for the Gelatin Medium. The addition of gelatin affects the stiffness of the medium, which in turn affects the properties of BC synthesized within it. As detailed in the previous section, the indentation load was measured at a fixed indentation depth of 1 mm and used as an indicator of the relative stiffness of the gelatin medium. This indentation load-based stiffness indicator was seen to increase as a function of the gelatin concentration with values of 2.4 ± 1.61 N, 6.2 ± 0.78 N, and 10 ± 1.73 N for 2.5, 5, and 7.5 wt % of gelatin, respectively. While the 0.1 wt % gelatin sample was excluded due to the limitation of load cell sensitivity, the trend of increase in the relative stiffness of the medium with an increase in gelatin concentration is consistent with literature.[48]

Changes in BC Production Metrics and Material Properties. Table 1 outlines the general production metrics and material properties of the BC pellicles as a function of gelatin concentrations. Even low concentrations of gelatin (0.1 wt %) resulted in substantial changes in BC matrix formation (Table 1). The productivity ($g{\cdot}L^{-1}h^{-1}$) of BC pellicle formation increased by 390% between gelatin concentrations of 0 and 2.5 wt %, reaching a value of 1.64. Further increases in gelatin concentration to 5 and 7% resulted in decreases in BC pellicle formation productivity. It follows that the maximum carbon conversion efficiency (CCE) is at 2.5 wt % gelatin. That is, the %-CCE during pellicle formation increased by 1.6-fold for gelatin concentrations of 0 and 2.5 wt %, respectively, reaching 27.1%. Further increases in gelatin concentration to 5 and 7% resulted in decreases in %-CCE. In fact, the CCE at 7.5 wt % gelatin was 55% of that with 0% gelatin. Thus, higher productivity corresponds to increased %-CCE.

At 5 wt % gelatin in the cultivation medium, the BC matrix sample density relative to that of the control decreased, by 15-fold, to 0.014 g/cm^3. At all concentrations of gelatin, from 0.1 to 7.5%, the density was lower than that of the control. Hence, inclusion of gelatin in cultivation media of *K. xylinus* ATCC 700178 resulted in BC matrices with a higher free volume. At

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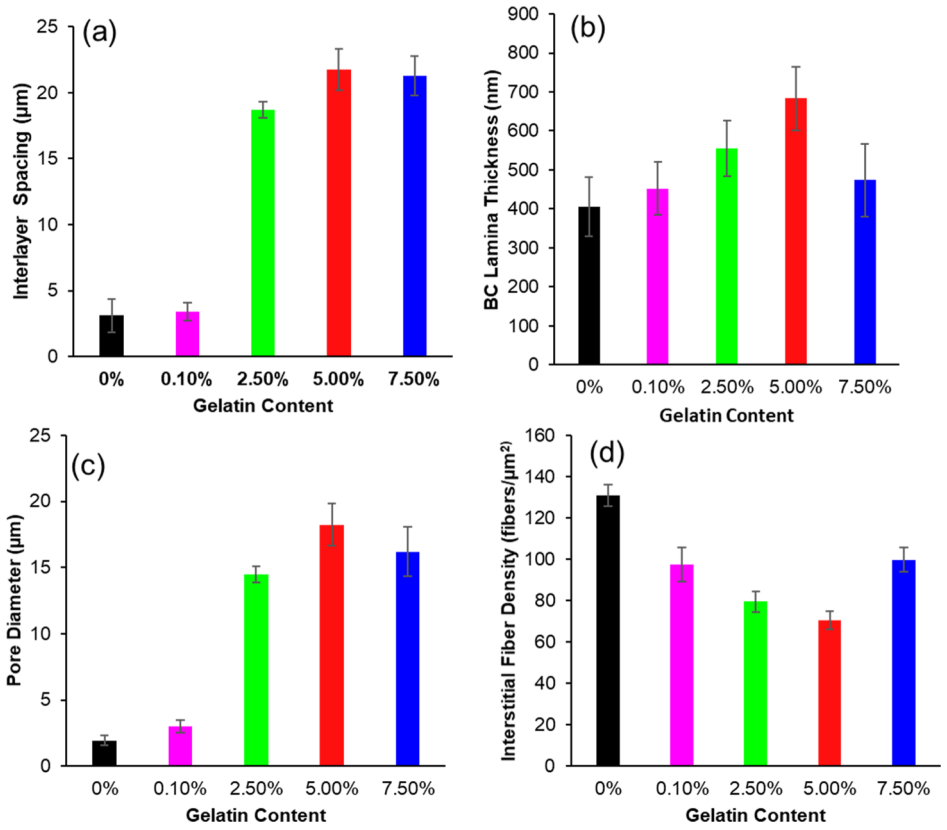

Figure 3. Measured morphological characteristics from BC microstructure images. (a) Interlayer spacing, (b) BC lamina thickness, (c) pore diameter, (d) interstitial fiber density.

gelatin concentrations up to 5%, the BC pellicles remained highly crystalline (92.2−94.6%) (Table 1). However, at 7.5 wt % gelatin, the BC matrix %-crystallinity decreased to 85.6%, which may correspond with the physical disruption observed in the BC matrix at 7.5 wt % gelatin (see Figure 2). Water content analysis revealed that, at 5 wt %-gelatin in cultivations, the water content in the matrix reached its highest value (306.6 g per g BC). This represents a 3-fold increase relative to that of the control (Table 1). The trends in matrix water content followed that of density values. Similarly, BC matrix dry wt. followed the trends in productivity, reaching maximum values at 2.5 wt % gelatin. Also, the dry thickness of cleaned and lyophilized BC matrices increased 20-fold relative to that of the control at 5 wt % gelatin. The trends in film thickness as a function of %-gelatin follow those of reductions in density and increases in water content (Table 1).

Microstructure Characterization. Cross-sectional views of BC matrices were obtained from SEM images. As seen in Figure 1, the BC synthesized in the presence of gelatin demonstrated a stratified microstructure, revealing the formation of laminae (layered structures) connected by interstitial fibrils. The microstructural features of interest include the following: (i) interlayer spacing (S in Figure 1) denoting the spacing between two consecutive lamina; (ii) pore diameter (d) denoting the diameter of the pores formed between interstitial fibers and BC laminae (shown by a green circle in Figure 1); (iii) BC lamina thickness (t in Figure 1) denoting the thickness of the BC lamina; and (iv) interstitial fiber density (fibers/μm^2), quantified by counting the number of fibers in a fixed window of 3 μm × 50 μm (n = 10).

As seen in Figure 2b−e, a stratified morphology (i.e., layered organization) is observed in the meso-scale SEM cross-sectional images of BCs formed with 0.1, 2.5, 5, and 7.5 wt % gelatin in cultivations. BC formed without gelatin, representing the control, has remnants of stratification that lack directional order (Figure 2a). Furthermore, major disruptions in the stratified morphology are evident at 7.5 wt % in Figure 2e.

Important differences present in the stratified microstructures are revealed by the degree of alignment of the dense BC layers. To quantify the stratified morphology seen in the SEM images, directionality histogram curves were extracted for the BC laminae images in Figure 2a−e using ImageJ. Representative directionality histogram curves are presented in Figure 2f. The mean of the histogram curves in Figure 2f indicates the dominant orientation of the BC laminae within the microstructure. The spread of these histogram curves defines the degree of deviation of the BC laminae from its dominant orientation. This spread is observed to decrease from the control sample up to that prepared with 5 wt % gelatin, indicating improved directionality of the BC laminae. Increasing the gelatin concentration to 7.5 wt % results in an increased spread of the curve, which captures the disorder seen in Figure 2e.

In addition to directionality, the morphological features presented in Figure 1 were also measured for samples synthesized at different gelatin contents. The corresponding values, presented in Figure 3a−d, provide insights on how the amount of gelatin in the bacterial culture medium affects the key morphological features. The interlayer spacing (Figure 3a) did not significantly change at 0.1 wt % gelatin relative to that

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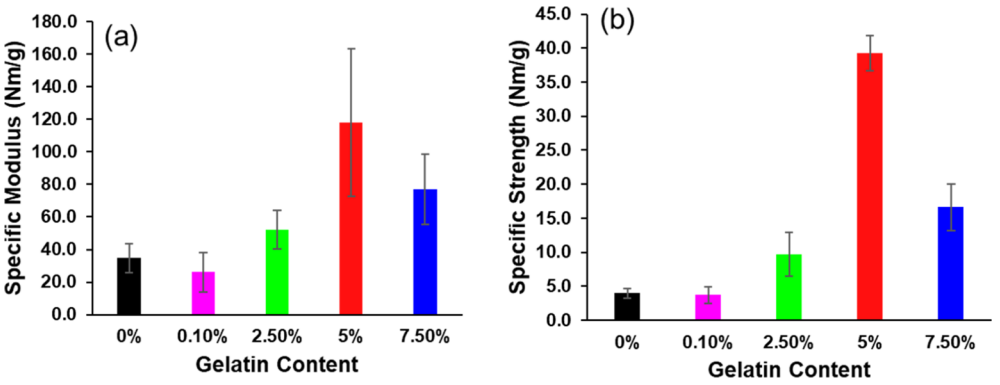

Figure 4. Summary of BC tensile testing results. (a) Specific modulus of BC pellicles; (b) specific strength of BC pellicles vs. the gelatin content in the synthesis medium. The values listed are the mean and standard deviation from at least five samples.

of the control. However, increase in the gelatin concentration from 0.1 to 2.5 wt % resulted in a 5.5-fold increase in the interlayer spacing. The highest values of interlayer spacings are at 5 and 7.5 wt % gelatin (~20 μm).

The average thickness of the BC laminae tends to increase with the wt % gelatin in the cultivation medium by up to 5% (Figure 3b). However, large values of standard deviation result in statistically insignificant changes from the control to 2.5 wt % gelatin. Nevertheless, at 5 wt % gelatin, the thickness of BC laminae increased to 683.4 ± 82.1 nm, which is significantly greater than the values of the control and 0.1 wt % gelatin (405.2 ± 76.4 nm and 451.6 ± 67.9 nm, respectively). Further increase in the wt % gelatin to 7.5 wt % resulted in a significant decrease in the BC laminae thickness.

The average pore size diameter increased by about 9-fold at 5 wt % gelatin relative to that of the control, reaching 18.23 μm (Figure 3c). In fact, increasing the gelatin content from 0.1 to 5 wt % resulted in a regular increase in pore diameter. However, further elevation of the wt % gelatin from 5 to 7.5 wt % results in an 11% decrease in the mean pore size.

Plotted values of the interstitial fiber density between BC laminae are displayed in Figure 3d. From the control to 5 wt % gelatin, the fiber density decreases by 46% to 70.5 fibers/μm^2. Comparison of Figure 3a,d shows that the decrease in the interstitial fiber density is accompanied by an increased distance between the BC laminae (interlayer spacing). In other words, increasing gelatin concentration in culture media causes an inverse trend between the interlayer spacing (Figure 3a), which increases, and interstitial fiber density, which decreases (Figure 3d).

Tensile Properties of BC Pellicles. Tensile testing was performed on BC matrices after the removal of cells, gelatin, and remaining nutrient media components as described in the previous section. The sample orientation ensured that the tensile load was applied parallel to the BC laminae direction. Stress−strain curves similar to those reported in Amason et al.[43] were obtained from load−displacement data. The heterogeneity of the pellicle in terms of microstructure was addressed by comparing the specific strength and specific modulus of the samples. The specific metrics consider the changes in density/fibril arrangement in BC samples and allow comparison between samples that are microstructurally heterogeneous. The specific modulus and specific strength values were obtained by normalizing the modulus and the ultimate tensile strength obtained from the engineering stress−strain curves by the corresponding density values reported in Table 1.

As seen in Figure 4a,b, both the specific modulus and specific strength values show a similar trend in that both properties show a value increase from the control sample (0 wt % gelatin) to 5.0 wt % gelatin, beyond which there is a drop at 7.5 wt % gelatin. The trend in mechanical properties mirrors the trend in microstructural morphology as indicated in Figure 3a−c. This would suggest that the varying morphological features discussed in the previous section are responsible for the change in the mechanical properties of BC matrices.

DISCUSSION

Changes in BC Physical Metrics Caused by Gelatin. The addition of gelatin and other polymeric additives to BC culture media resulted in differences in the physical characteristics of the ensuing BC matrix.[21,22,37,38] Herein, the productivity of BC formation increased from the no-gelatin control, at 0.42, to 1.64 g·L^{-1}h^{-1} and 1.29 g·L^{-1}h^{-1} at 2.5 and 5 wt % gelatin, respectively (Table 1). However, at the highest tested gelatin concentration (7.5 wt %), the productivity decreased to 0.56 g·L^{-1}h^{-1}. A similar trend was observed by Taokeaw et al.[37] with the addition of gelatin beyond 5%.

We believe that the presence of gelatin in culture media causes BC generating bacteria to remain at the air−water interface with minimal fluctuations in their position for longer periods, leading to a higher BC production. This is a consequence of the stiffening effect of gelatin discussed above due to which cell mobility is increasingly restricted relative to the liquid media of the control sample. This increased time spent by the bacteria at the interface is hypothesized to increase the carbon conversion efficiency (CCE), that is, the effectiveness of the bacteria to generate cellulose from mannitol, the carbon source. The change in CCE correlates with the change in productivity that rises as the gelatin concentration increases, reaches a maximum, and then decreases at relatively higher gelatin concentrations. These changes, as listed in Table 1, occur with a maximum of 27.1% CCE at 2.5 wt % gelatin, an increase of 1.6-fold relative to that of the control. Both metrics of CCE and BC production increase proportionally up to 2.5 wt % gelatin. For gelatin loadings above 2.5 wt %, decreases are observed in both CCE and BC productivity. Work by Pleass[49] in 1928, and Jordan-Lloyd[50] in 1931, indicates that increasing gelatin concentration leads to a decrease in free water in the gel medium that is accompanied by a reduced oxygen diffusion coefficient. We

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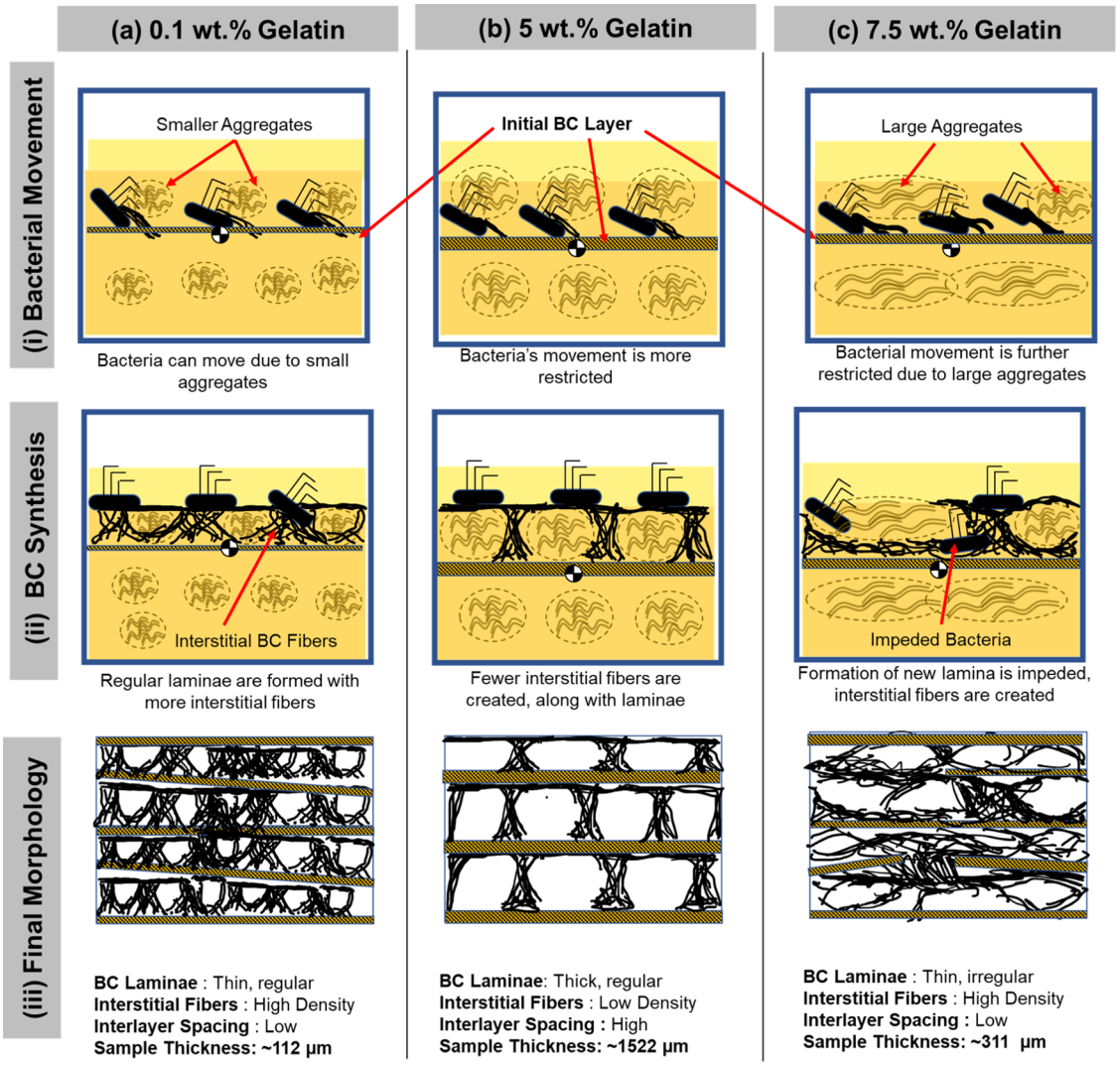

Figure 5. Mechanism of BC synthesis in (a) 0.1 wt %, (b) 5.0 wt %, (c) 7.5 wt % consisting of three phases: (i) bacterial movement within media, (ii) BC synthesis within media, and (iii) microstructure obtained at the end of culture.

suspect that, at 5.0 and 7.5 wt % gelatin, this reduction in dissolved oxygen dominates over decreased cell mobility, resulting in decreases in CCE and productivity metrics.

Increases in BC productivity up to 2.5 wt % gelatin directly correlate with increases in the grown BC dry weight and dry thickness. The dry weight of the matrix was measured after water removal by lyophilization that largely preserves the microstructure for further analysis.[51,52] Some physical characteristics of the formed BC matrix saw maxima at 5 wt % gelatin. Specifically, the dried BC matrix thickness is 2251.1 μm at 5 wt % gelatin (Table 1). However, further increase in gelatin concentration to 7.5 wt % caused a large decrease in matrix thickness to 311.6 μm. Maximum BC matrix thickness correlates with maxima in interlayer spacing distance (~22 μm), and corresponding pore diameter (~18 μm), that also occur at 5 wt % gelatin.

While the control BC density is 0.206 g/cm^3, the density decreased to 0.014 g/cm^3 at 5 wt % gelatin (Table 1). This 3.5-fold overall decrease in BC matrix density correlates with increases in interlayer spacing distance and pore size described above (Figure 3). Furthermore, these changes in matrix parameters enable BC matrices to hold higher amounts of water per unit weight of BC. Indeed, the water content increases from 99.2 g for the control to 306.6 g for the 5 wt % gelatin. This is an extraordinary increase in matrix porosity that can be of great value for applications such as skin burn treatments that require diffusion of active ingredients from the BC matrix to the wounded areas. A discussion of changes in matrix morphology, such as the interlayer spacing and pore size, is presented further below.

One concern with experimental parameters that cause physical changes in the BC matrix is that such changes may come at the cost of beneficial properties such as BC crystallinity. However, inclusion of gelatin in BC formation cultivation medium up to 5 wt % did not result in substantial changes in %-crystallinity, which remained at ~92−94%. Nevertheless, further increase in the gelatin concentration to 7.5 wt % did result in a reduction to ~86% crystallinity (Table 1). A reduction in BC matrix crystallinity at relatively high gelatin concentration was also observed by Taokeaw et al.[37] This reduction in crystallinity is likely explained by the fact that the process of self-assembly of single microfibers extruded from the cell wall to nanoribbons is disturbed by the increased stiffness measure of the 7.5 wt % gelatin medium, which is ~61% greater than that of the 5 wt % gelatin medium. This is

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also consistent with the morphological irregularities observed at 7.5 wt % gelatin (Figure 2).

Gelatin Effects on BC Microstructure Morphology and Mechanical Properties. The addition of gelatin to the nutrient media affects the microstructural morphology of synthesized BC. Gelatin addition leads to a stratified microstructure, with distinct BC laminae and interstitial fibers in between the laminae (Figure 1). The amount of gelatin also affects key morphological characteristics in the stratified structure, viz., BC lamina thickness, interlayer spacing, pore diameter, and interstitial fiber density. The mechanism hypothesized here for the effect of gelatin addition is an interplay of multiple factors using the illustration displayed in Figure 5. The stiffness, characterized by indentation load measurements above, shows the extent that medium stiffness increases with increased gelatin concentration. However, we will first discuss the effect of increasing gelatin weight percentages up to 5 wt %. Following this, the 7.5 wt % case will be discussed separately.

Explanation of the Influence of the Gelatin Content on BC Morphology. Gelatin, when combined with low-molecular-weight molecules such as sugars and sugar alcohols present in the nutrient media, forms small aggregates in the microstructure upon gelation. These aggregates, estimated in previous work by light scattering studies to be about 2 μm in diameter for 6 wt % gelatin, are formed as they combine with small molecules in the media such as sugars.[34,53,54] While the size of these structures has not been characterized for the gelatin media used in this work, it should increase with the increased gelatin content in the media, primarily due to the increased hydrophobic interactions between gelatin chains, which leads to aggregate formation.[53]

The BC laminae are synthesized by the bacteria at the air−nutrient interface (Figure 5a). The presence of gelatin aggregates in the media results in increased stiffness, which in turn restricts cell motility and, consequently, increases cell residence time at the air−water interface. In other words, cells move more slowly along the $X-Y$ plane of the air−water interface. Assuming continuous BC production, this slowed cell mobility results in a larger amount of in-plane BC deposition for gelatin loadings up to 5 wt %. This explains the increase in the thickness of the BC laminae from 405.2 nm for the control to 683.4 nm at 5 wt % gelatin (Figure 3b). As the initial BC lamina forms (Figure 5, top row (i)), the bacteria can replicate and are driven to rise back to the air−nutrient media interface where oxygen is readily available, and the formation of the next dense layer begins. For gelatin loadings up to 5 wt % this process continues as BC formation progresses layer-by-layer.

Another consequence of increased gelatin concentration is an increase in the size and frequency of gelatin aggregates. Figure 5a,b,c, respectively, depicts changes in frequency and size of gelatin aggregates for gelatin concentrations of 0.1, 5, and 7 wt %. This figure also illustrates how, with increased gelatin concentration up to 5 wt %, aggregates become increasingly large, thereby occupying more space between cells. Consequently, the distance between laminae and the pore size increases, while the interstitial fiber density decreases. Furthermore, the frequency of cell rotation is less often and slower at higher gelatin concentrations, further resulting in larger pores.

When the gelatin concentration is increased from 5 to 7.5 wt %, the corresponding increases in gelatin aggregate size and medium viscosity become disruptive to BC formation, causing misalignment of cells (Figure 5c). Its effect on the BC morphology is captured by the increase in the spread of the directionality histogram in Figure 2f, which implies higher diversity in alignments of BC laminae. This disturbance in directionality results in (a) decreased thickness of the laminae as cells become temporarily detached and (b) higher density of interstitial fibers. Furthermore, misalignment of cells and large aggregates impedes dense fiber formation to an extent that causes discontinuities along laminae.

Implications of Gelatin on BC Mechanical Properties. The varying concentration of gelatin affects key microstructural characteristics as detailed in Figure 3 and represented in Figure 5. The mechanical properties, viz., the specific modulus and specific strength, are plotted in Figure 4. In BC, the tensile load is distributed between two distinct microstructural components, viz., the BC laminae and the interstitial fibers. The trends seen in the thickness of the BC lamina (Figure 3b) and the interstitial fiber density (Figure 3d), as a function of gelatin content, explain the trends in the mechanical properties reported in Figure 4.

For gelatin loadings between 0 and 5 wt %, the number of interstitial fibers decreases as a function of increased gelatin concentrations, whereas simultaneously an increase is seen in the thickness of the BC laminae. This implies that, as the gelatin concentrations are increased, a greater percentage of the tensile load in the elastic region is sustained by the BC laminae as opposed to the interstitial fibers. Correspondingly, one observes the specific modulus values to increase because of the relatively high packing density of BC fibers within these dense laminae.

The increase in specific strength of the BC sample for gelatin loadings between 0 and 5 wt % is attributed to two factors, viz., (a) the increase in the thickness of BC laminae as seen in Figure 3b and (b) the increase in alignment of BC laminae as indicated by the decreased spread in the directionality histograms in Figure 2f. Increases in the laminae thickness (dimension) improve the load bearing capacity of each BC lamina. Furthermore, the increased regularity indicates that more BC laminae are oriented in the direction of the load application. This combination of highly oriented laminae with a larger thickness allows the sample to sustain larger loads at high strains, thereby resulting in an increase in its specific strength.

The change in morphological characteristics between 0.1 wt % gelatin and the control sample is not significant, and the same trend is reflected in its specific strength/modulus (Figure 4). From 2.5 to 5 wt % gelatin, there is a significant increase in BC lamina thickness and the regularity/alignment of the BC laminae, which also corresponds to the trends in the specific strength/modulus. The drop in specific modulus/strength of the sample synthesized at 7.5 wt % gelatin is also mirrored in Figure 3, where the increased gelatin content disrupts the layered structure leading to a loss of strength that is attributed to the presence of a regular layered structure. The disruption in matrix regularity leads to loss in mechanical integrity of the sample.

CONCLUSIONS

This study compares microstructural and physical properties of BC grown in a traditional medium (HS broth) and those grown with a stiffness altering and template forming additive, gelatin. The use of gelatin as a pore forming and production

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increasing additive is not new, however, determining the effects of gelatin concentration on BC matrix mechanical properties along with detailed characterization of morphological parameters (e.g., spacing between dense layer, regularity of that spacing, thickness of dense fiber layers, and the number of fibers per unit area between dense layers) has not been described and interpreted with respect to media characteristics. Mechanical properties were determined after gelatin removal, enabling comparisons in innate, BC matrix property changes. Based on our findings, gelatin, when added between the ranges of 2.5 to 5 wt %, provides the largest changes in BC matrix morphological parameters. Including 2.5 wt % gelatin in cultivation media results in the largest BC productivity, CCE values, and dry weight. Doubling the gelatin concentration to 5 wt % gave BC matrices with the lowest density and corresponding highest water holding capacity, dry thickness, and pore size. Also, at 5.0 wt % gelatin, the BC matrix formed reached the highest values of specific modulus and tensile strength which correlated with the largest values of interlayer spacings and dense layer thickness but the lowest number of fibers per unit area between dense layers. This ability to fine-tune matrix parameters was interpreted based on corresponding changes in cell mobility as a function of medium stiffness and the templating effect of gelatin that forms aggregated triple helices. Furthermore, reduction in the free volume of matrices as well as decreased availability of oxygen and nutrients with increased %-gelatin was used to explain the complex changes in media physical characteristics that result as a function of gelatin concentration.

A better understanding of gelatin effects on BC formation will require additional characterizations that can further tease out the complex set of variables that occur upon introduction of gelatin in BC cultivations. Important questions include what changes in gelatin triple helical aggregation occur across the concentrations studied and how cells and BC dense layers interact with gelatin aggregates. Furthermore, quantitative changes in cell mobility and the extent they are confined at surfaces will allow the construction of models to better explain the results obtained herein. Moreover, an in-depth study into how cell mobility and productivity are related, given the extraordinary increase in productivity revealed herein (0.42 g·L^{-1}·h^{-1} to 1.64 g·L^{-1}·h^{-1}), is needed. In future work, we will focus on characterizations that are needed to answer these important questions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04820.

Photographs and FTIR spectra of BC films prepared with 0 and 5% gelatin in the medium (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. The bacterial cellulose (BC) production-related materials, protocols, and characterization including microstructure were conducted by A-C.A., S.R., N.L., and G.S., supervised by R.A.G. The mechanical characterization efforts (i.e., gelatin indentation and BC tensile tests) and extraction of directionality histograms were conducted by A.M. and supervised by J.S. The final manuscript preparation including key figures was handled by A.M. under supervision from R.A.G and J.S. All authors have given approval to the final version of the manuscript.

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Notes

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