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Bacterial Cellulose Production and Its Structural Characterization

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Abstract

Bacterial cellulose (BC) is an extracellular biopolymer of Acetobacter xylinum, which is now known as Komagataeibacter xylinus. The polymer can be characterized as having outstanding purity, lacking lignin and hemicellulose, while exhibiting high crystallinity, superior mechanical strength, and a distinctive three-dimensional nanofibrillar network compared to plant cellulose. In the current study, BC was grown by inoculating a native isolate of K. xylinus in Hestrin–Schramm (HS) medium and incubating without stirring at 30°C over a period of 7 to 10 days. The gelatinous pellicle was created on the air-liquid interface due to the biosynthesis of bacteria.

The collected pellicles underwent an alkaline purification procedure in order to remove the rest of the bacterial cells and medium constituents. Purification was done by placing the sample in 0.5N sodium hydroxide (NaOH) then washing the sample with distilled water several times until a neutral pH was obtained. This procedure ensured the production of high-purity BC membranes.

Structural characterization was done to prove the successful synthesis of BC. The analysis by Fourier Transform Infrared Spectroscopy (FTIR) showed the typical cellulose functional groups (hydroxyl (-OH) and glycosidic (C-O-C) bonds) which are associated with high-purity cellulose. Scanning Electron Microscopy (SEM) showed a dense, interconnected nanofibrillar network with a large surface area, indicative of well-formed BC. Taken together, the findings indicate that high-quality bacterial cellulose could be effectively produced and purified within the local laboratory setting using cost-effective measures, which explains why this bacterial cellulose could be used in environmental cleanup and adsorption research.

Keywords: Bio-cellulose, Komagataeibacter xylinus, Hestrin–Schramm, FTIR

إنتاج السليلوز البكتيري وخصائصه البنيوية

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المستخلص

السليلوز البكتيري (BC) هو بوليمر حيوي خارج خلوي تُنتجه بكتيريا حمض الخليك، وخاصةً بكتيريا Komagataeibacter xylinus (المعروفة سابقًا باسم Acetobacter xylinum). يتميز السليلوز البكتيري عن السليلوز النباتي بنقائه العالي، إذ يخلو من اللجنين والهيميسليلوز، بالإضافة إلى بلوريته العالية، وقوته الميكانيكية، وبنيته الشبكية النانوية الليفية ثلاثية الأبعاد الفريدة. في هذه الدراسة المحلية، تم إنتاج السليلوز البكتيري باستخدام عزلة محلية من بكتيريا K. xylinus، تمت زراعتها في وسط هيسترين-شرام (HS) تحت حضانة ثابتة عند 30 درجة مئوية لمدة 7-10 أيام. تشكلت طبقة رقيقة من السليلوز الهلامي عند السطح الفاصل بين الهواء والسائل نتيجةً للتخليق الحيوي البكتيري.

تم جمع الطبقات الرقيقة المُستخلصة بعناية، وخضعت لعملية تنقية قلوية لإزالة الخلايا البكتيرية ومكونات وسط الزرع المتبقية. أُجريت عملية التنقية باستخدام هيدروكسيد الصوديوم (NaOH) بتركيز 0.5 مولار، تلاها غسل متكرر بالماء المقطر حتى الوصول إلى درجة حموضة متعادلة. ضمنت هذه المعالجة إنتاج أغشية السليلوز البكتيري عالية النقاء.

أكدت دراسة التركيب البنيوي نجاح إنتاج السليلوز البكتيري. كشف تحليل طيف الأشعة تحت الحمراء بتحويل فورييه (FTIR) عن مجموعات وظيفية مميزة للسليلوز، بما في ذلك مجموعات الهيدروكسيل (–OH) والروابط الجليكوسيدية (C–O–C)، مما يتوافق مع السليلوز عالي النقاء. أظهر التصوير المجهري الإلكتروني الماسح (SEM) بنية شبكية كثيفة ومترابطة من الألياف النانوية ذات مساحة سطحية عالية، مما يدل على جودة تكوين السليلوز البكتيري. تُظهر هذه النتائج مجتمعةً إمكانية إنتاج السليلوز البكتيري عالي الجودة وتنقيته بكفاءة عالية في ظروف المختبر المحلية باستخدام إجراءات فعالة من حيث التكلفة، مما يُبرز إمكانية استخدامه في معالجة البيئة ودراسات الامتزاز.

الكلمات المفتاحية: السليلوز الحيوي، كوماغاتاييباكتر زيلينوس، هيسترين-شرام، مطيافية الأشعة تحت الحمراء بتحويل فورية

Introduction

All plants contain cellulose, one of the most prevalent polymers in nature, and a vast range of microbes create it [1]. Certain bacterial strains have the ability to create extracellular cellulose, which takes the form of fibers that are affixed to the bacterial cell, in addition to cellulose that is taken from plant cells [2].

Other sources of cellulose production besides plants include fungi, seaweed, and a few bacterial organisms, particularly the aerobic non-pathogenic ones like gram-negative Komagataeibacter sp. that can produce cellulose and the Komagataeibacter xylinus isolate, formerly known as Acetobacter xylinum, which is currently the most studied species [3].

Bacterial cellulose is a unique biomaterial for biomedical usage because of its important macromolecular and surfactant properties, which make it perfect for both in vitro and in vivo medical and biological objectives [4].

Surface charge, wettability, topographical properties, and hydrophobic or hydrophilic constituents are ideal physical, chemical, and biochemical characteristics that are compatible with these interactions. The way cells respond to bioactive molecules determines the overall outcome of new biomedical device types [5].

Bacterial cellulose (BC) is a highly pure extracellular biopolymer produced by certain acetic acid bacteria such as *Komagataeibacter xylinus*. Unlike plant-derived cellulose, BC lacks lignin and hemicellulose and exhibits superior physicochemical properties including high crystallinity, a three-dimensional nanofibrillar network, excellent mechanical strength, and remarkable water-holding capacity [6]. These unique characteristics, along with its extensive surface area and abundant hydroxyl groups, make BC an attractive material for biomedical, environmental, and industrial applications.

The yield and structural properties of BC are strongly influenced by the bacterial strain used and the cultivation conditions, including medium composition and fermentation parameters [7]. Locally isolated strains and optimized culture conditions can therefore play a crucial role in enhancing BC productivity and quality for laboratory or industrial applications.

Objectives of the Study

Using a local strain of *Komagataeibacter xylinus* (formerly *Acetobacter xylinum*), the study sought to manufacture, purify, and characterize bacterial cellulose while maximizing fermentation and

purification conditions to create high-quality cellulose appropriate for adsorption and environmental applications.

Materials and Methods

Bacterial cellulose (BC) was produced using the locally identified Acetobacter xylinum (Komagataeibacter xylinus) under static fermentation. The modified Hestrin–Schramm (HS) medium [8] was prepared as follows per liter of distilled water: sucrose 10 g, peptone 2.5 g, yeast extract 2.5 g, Na_2HPO_4 1.35 g, citric acid 0.6 g, acetic acid 1 mL, and ethanol 2.5 mL. The medium was sterilized by autoclaving at 121°C for 15 min

The bacterial inoculum was added to the sterilized medium, and cultures were incubated at 28–30°C without agitation for 7–14 days to allow formation of gelatinous BC pellicles at the air–liquid interface. The pellicles were harvested, [9] rinsed with distilled water, and purified using 0.5–1.0 M NaOH for 1–2 hours to remove cells and impurities, followed by repeated washing until neutral pH. Purified BC films were deemed suitable for subsequent characterisation. The wet mass of each film was measured immediately after removal from the culture medium using an analytical balance. The length of each film was measured using a calibrated ruler. The dry mass of the BC was measured after air-drying it at ambient temperature (48 h). According to Singhsa et al. [10], the measurements provide the opportunity to consider the water retention capacity and evaluate the reliability and stability of BC production under the experiment conditions used Statistical analysis SPSS software was used for statistical analysis. Values were expressed as mean standard deviation (SD) of six independent measurements (n = 6) As shown in Table (1).

Table (1): Physical characteristics of bacterial cellulose produced under static fermentation conditions

Parameter	Minimum	Maximum	Mean ± SD	n
Wet weight (g)	25.82	27.33	26.53 ± 0.60	6
Length (cm)	7.00	9.40	8.97 ± 0.97	6
Dry weight (g)	0.13	0.15	0.14 ± 0.009	6

Values are expressed as mean ± standard deviation (SD) based on six independent measurements (n = 6) .

Results

Wet weight of bacterial cellulose pellicles was 26.53 0.60 g, and the average length was 8.97 0.97 cm. The dry weight was much lower than that of the dry weight, at a mean value of 0.14 0.009 g. All measurements were performed in six replicates (n=6) and showed good reproducibility with low standard deviation values.

Figures of the wet weight, length and the dry weight of bacterial cellulose pellicles synthesized under promotional fermentation conditions are given as shown in Table (1). The wet weights ranged from 25.82–27.33 g, with the lowest and highest values being replicate 5 (25.82 g) and replicate 2 (27.33 g).

Pellicle length was 7.0–9.4 cm. The replicates tended to have a similar length (approximately 9.4

cm), suggesting a consistent growth of the surface at the air-liquid interface; replicate 5, however, had a significantly shorter length (7.0 cm), which could be explained either by some minor mechanical perturbation during incubation, heterogeneity of nutrient locally or low oxygen diffusion at the culture surface.

Dry weight measured between 0.13 g and 0.15 g. Since the dry weight indicates the true content of cellulose at the end of the process when it is fully dehydrated, these values prove that the tested isolate biosynthesizes BC successfully as shown in Figure (1). The low inter-replicate standard deviation also testifies to the uniformity of cellulose yield. The substantial disparity between wet and dry weights is indicative of the remarkable water-retention capacity of BC, which is directly attributable to the characteristic three-dimensional nanofibrillar network structure formed during biosynthesis.

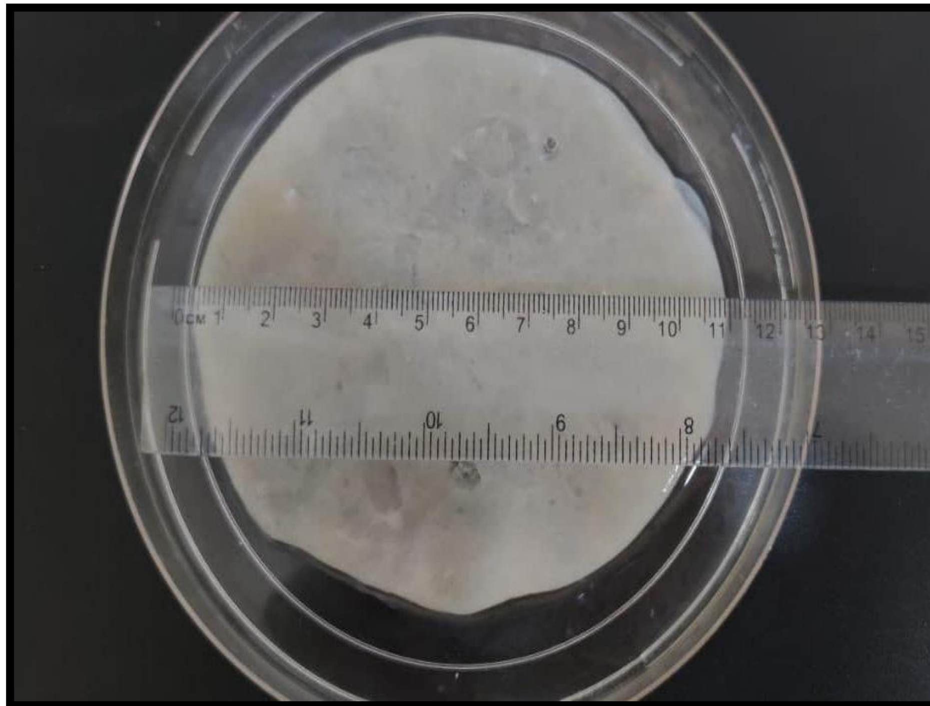
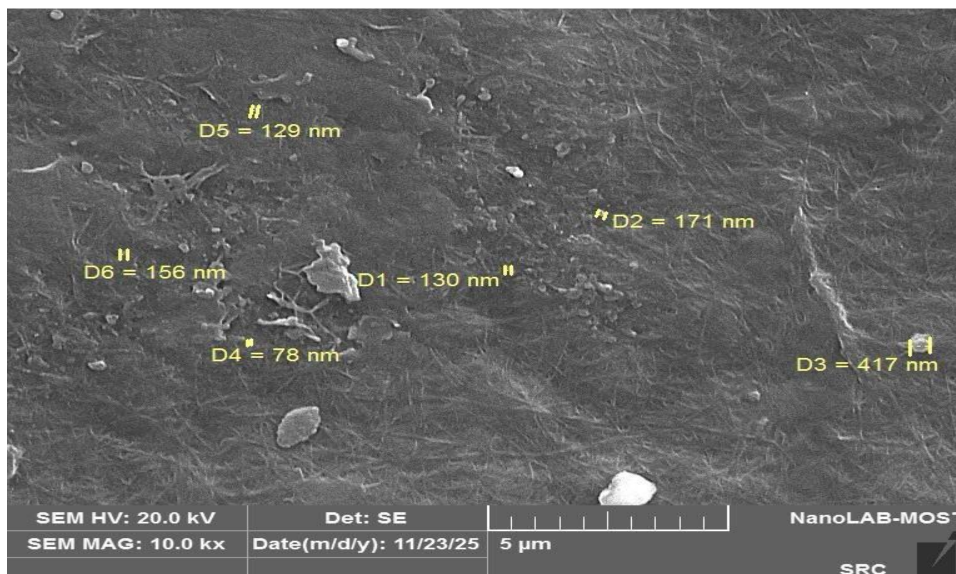
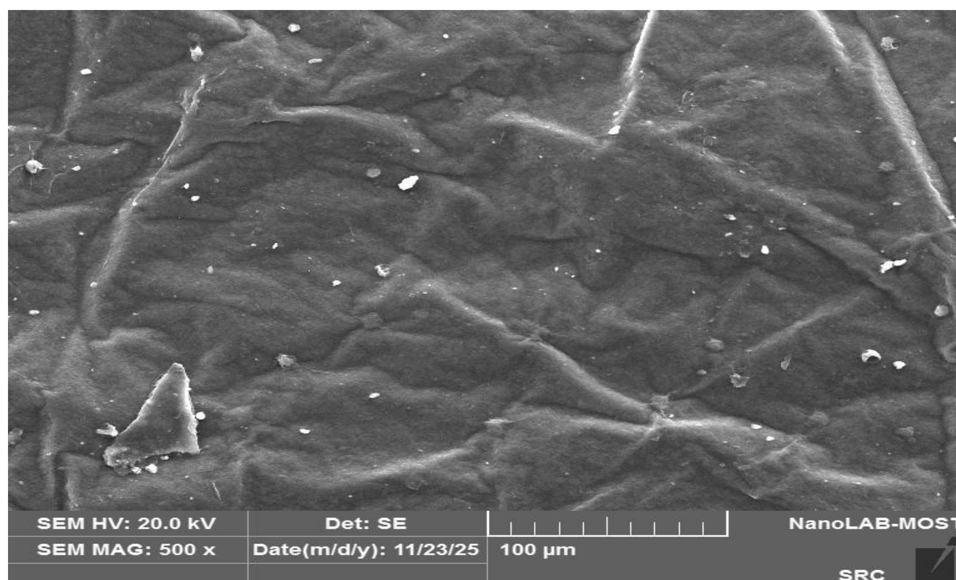


Figure (1) : Production of bacterial cellulose under typical conditions



A: SEM Micrograph Showing Fiber Morphology at a Scale of 5 µm

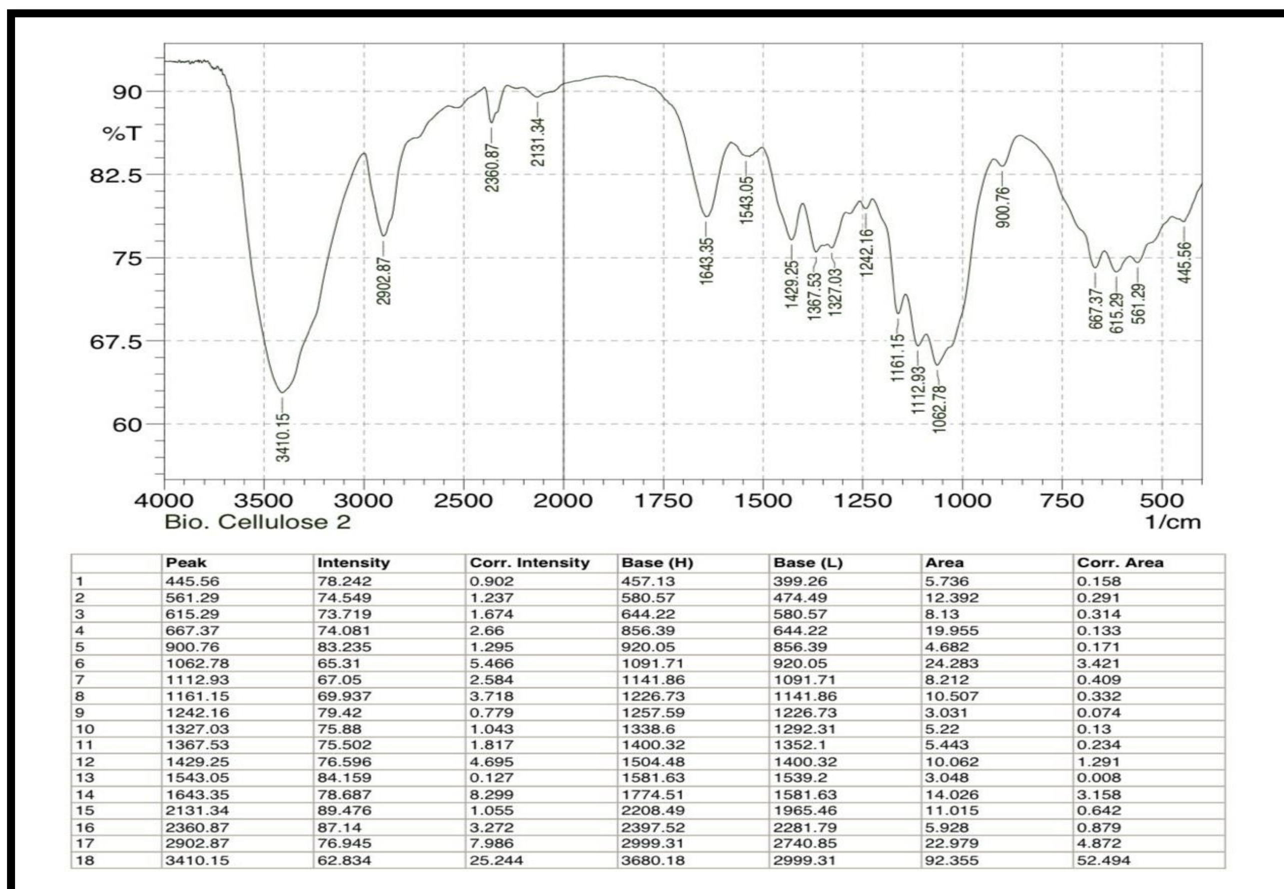


B: SEM Micrograph Showing Fiber Morphology at a Scale of 100 µm

Figure (2): A SEM Micrograph Showing Fiber Morphology at a Scale of 5 µm

B: SEM Micrograph Showing Fiber Morphology at a Scale of 100 µm

Figure (2) presents Scanning Electron Microscopy (SEM) images used to characterize the surface morphology of bacterial cellulose under ambient conditions . Using conductive carbon tape, dried samples were placed on copper stubs, sputter-coated with gold for 30 seconds, and viewed at 8,000×, 15,000×, and 20,000× magnifications to assess their surface characteristics [11]



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	445.56	78.242	0.902	457.13	399.26	5.736	0.158
2	561.29	74.549	1.237	580.57	474.49	12.392	0.291
3	615.29	73.719	1.674	644.22	580.57	8.13	0.314
4	667.37	74.081	2.66	856.39	644.22	19.955	0.133
5	900.76	83.235	1.295	920.05	856.39	4.682	0.171
6	1062.78	65.31	5.466	1091.71	920.05	24.283	3.421
7	1112.93	67.05	2.584	1141.86	1091.71	8.212	0.409
8	1161.15	69.937	3.718	1226.73	1141.86	10.507	0.332
9	1242.16	79.42	0.779	1257.59	1226.73	3.031	0.074
10	1327.03	75.88	1.043	1338.6	1292.31	5.22	0.13
11	1367.53	75.502	1.817	1400.32	1352.1	5.443	0.234
12	1429.25	76.596	4.695	1504.48	1400.32	10.062	1.291
13	1543.05	84.159	0.127	1581.63	1539.2	3.048	0.008
14	1643.35	78.687	8.299	1774.51	1581.63	14.026	3.158
15	2131.34	89.476	1.055	2208.49	1965.46	11.015	0.642
16	2360.87	87.14	3.272	2397.52	2281.79	5.928	0.879
17	2902.87	76.945	7.986	2999.31	2740.85	22.979	4.872
18	3410.15	62.834	25.244	3680.18	2999.31	92.355	52.494

Figure (3): FTIR analysis of dried bacterial cellulose produced by Komagataeibacter xylinus

BC-based functionalgroups were analyzed using the FTIR apparatus (see figure 3), which subsequently transformed into a pellicle when KBr powder was combined (1:5, BC: KBr). In order to investigate the 4000–400 cm−1 associated spectrum area, a sample of this mixture was obtained and employed in this test with a resolution of 6 cm−1 [12].

Table (2): The distinguish bands by FTIR analysis for dry bacterial cellulose produced by Komagataeibacter xylinus

Peak No.	position (cm^{-1})	Functional group	Observation
1	**445.6**	**C – C, C – O**	Structural vibrations of the cellulose chain
2	**561.3**	**O – C – O**	Distortional vibrations in the glucose ring
3	**615.3**	**C – C**	Internal vibrations of the cellulose structure
4	**667.4**	O–H out-of-plane bending	Deformation of hydroxyl groups associated with hydrogen bonds
5	**900.8**	β (1→ 4) glycosidic ring vibration	Cellulose fingerprint
6	**1062.8**	**C – O stretch (primary alcohol)**	C–O in C6
7	**1112.9**	C – O stretch (secondary alcohol)	crystalline cellulose
8	**1161.2**	C – O – C asym stretch (bridging)	glucosidyl bond
9	**1242.2**	C – O – H bending	In cellulose/hydroxyl
10	**1327.0**	**CH_2 twisting**	Indication of chain organization
11	**1367.5**	**C – H wag (O–H in-plane)**	Distinctive cellulose pattern
12	**1429.3**	**CH_2 scissoring**	Sensitive to cellulose crystallization
13	**1543.1**	**O–H Weak bending**	Non-characteristic of cellulose; likely instrumental noise.
14	**1643.4**	**H–O–H bend**	Water absorbed into the sample
15	**2131.3**	**CO_2**	Background/atmosphere, non-structural
16	**2360.9**	**CO_2**	Background/atmosphere, non-structural
17	**2902.9**	**C–H stretch**	CH/CH2 groups in cellulose
18	**3410**	**O–H stretch**	Hydrogen bonds

FTIR Spectral Analysis of Bacterial Cellulose

The use of Fourier-transform infrared (FTIR) spectroscopy corroborated the effective biosynthesis of chemically pure bacterial cellulose (BC). Extensive hydrogen bonding was revealed by a wide O-H spectrum at 3410 cm^{-1}, and the presence of ~900 cm^{-1} was a sure indication of the β(1→4) glycosidic cross-linkages prevalent in cellulose. The lack of absorption between 1700–1750 cm^{-1} confirmed the absence of any carbonyl groups hence proving chemical purity and absence of oxidation or esterification. Typical C-O and C-O-C vibrations were observed in the region of 1000–1160 cm^{-1} and the band at 1643 cm^{-1} indicated adsorbed water, which is in line with the hydrophilic nature of BC and high capacity of holding water.

Discussion

1. BC Production Yield: Wet Weight, Dry Weight, and Pellicle Dimensions

During the production of bacterial cellulose (BC) with the Komagataeibacter xylinus strain locally isolated under static fermentation conditions in Hestrin-Schramm (HS) medium, gelatinous pellicles with homogenous physical properties were obtained over six replicates (Table 1). Wet weights were 25.82–27.33 g, dry weights were 0.13–0.15 g, and pellicle lengths were 7.0–9.4 cm. This inter-replicate consistency is a sign of reproducible and constant fermentation environment. This is consistent with the findings of [6], who showed that static culture of K. xylinus in HS medium can generate well-developed BC pellicles with high water content and uniform fibrillar structure [7]. The wet-to-dry weight ratio is extremely high (approximately 180:1) in this study demonstrating what is referred to as the extraordinary water-holding capacity (WHC) of BC that is due to a combination of a complex three-dimensional nanofibrillar structure and the large number of hydrophilic hydroxyl (-OH) moieties on the cellulose polymer chains [13]. This exclusive ability to entrap and hold water molecules in its fibrillar structure is also reinforced by similar variations in wet and dry weights of BC pellicles grown using various K. xylinus strains [6].

Replicate 5 had slightly shorter pellicle length (7.0 cm) and the lowest wet weight (25.82 g) when compared to other replicates. This variation could be due to local disturbances of incubation like mechanical noise, uneven diffusion of oxygen or local heterogeneity in nutrient supply of the culture vessel. [14] reported that BC production under static culture conditions is highly sensitive to disruptions of the air-liquid interface, which is the major site of pellicle assembly [7]. Nonetheless, in spite of this small difference, general data similarity indicates the consistency of fermentation protocol reproducibility and proves the suitability of the locally isolated strain to generate BC under standard laboratory conditions.

2. Scanning Electron Microscopy (SEM) Analysis

Scanning electron microscopy (SEM) of the dried BC membranes (Figure 2) revealed a highly interconnected three-dimensional nanofibrillar network. Isolated nanofibrils had a uniform diameter and were randomly oriented creating an intricate web-like structure with a great porosity and specific surface area. Such a morphologic profile is quite consistent with the well-assessed ultrastructural profile of cellulose of bacterial origin, presented in the literature. [6] also found that BC manufactured by K. xylinus has a typical

nanofibrillar network, with fibrillar diameters ranging between 20–100 nm [7]. The porous, reticulated structure which is viewed in the present case is directly related to the high water-binding capacity and surface area of BC which are significant determinants of the binding capacity of this substance by their adsorption properties and filtration applications. Besides, the lack of observable cellular debris objects or condensed material in the SEM images gives visual evidence of the suitability of the alkaline purification process used, supporting the production of structurally pristine, highly pure BC membranes.

3. FTIR Spectral Analysis and Chemical Characterization

The FTIR spectrum of the formed BC (Figure 3 and Table 2) showed a series of typical absorption bands which all proved the successful biosynthesis of chemically pure cellulose. The observed broad and intense absorption at 3410 cm^{-1} is explained by O-H stretching of the cellulose polymer due to extensive intermolecular and intramolecular network of hydrogen-bonding which is one of the characteristic structural properties of the cellulose polymer. This band is regularly detected in FTIR spectra of both plants and bacterial-derived cellulose and is indicative of the large number of hydroxyl groups, contained in the crystallized cellulose lattice. The C-H stretching bands of cellulose would indicate a band at around 2903 cm^{-1}, which should be the C-H stretching vibrations of the methylene (CH_2) groups in the glucopyranose ring, which is in line with the aliphatic backbone of the cellulose chain.

Hydrophilic nature and high WHC of BC is fully supported by the presence of H-O-H bending of adsorbed water molecules at 1643 cm^{-1}, and consistent with the absorption spectrum of the product. The fingerprint area (1000–1160 cm^{-1}) displayed several overlapping bands that can be attributed to C-O and C-O-C stretching vibrations of the glucopyranose ring at C-1, C-2, C-3, C-5, and C-6, which indicates the presence of the cellulose repeat unit. Precisely, the band at 1062.8 cm^{-1} is attributed to the C-O stretching of the primary alcohol (C -6), and that at 1112.9 cm^{-1} to the C-O stretching of the secondary alcohol and is considered an indicator of crystalline cellulose. The asymmetric C-O-C stretching of the glycosidic bridge at a band at 1161.2 cm^{-1}, confirming the β(1→4) glycosidic linkage. Importantly, the band at 900.8 cm^{-1} of the well-known diagnostic fingerprint of cellulose type 1, which confirms the β-anomeric form of the glycosidic bond. The total lack of strong absorption in the 1700–1750 cm^{-1} region, which would otherwise contain carbonyl (C = O) absorption in case of an ester, aldehyde functional group, or carboxylic acid this indicates beyond reasonable doubt that there is high chemical purity and no oxidation or esterification of the product BC. These spectra are in excellent agreement with the reported FTIR spectra of BC synthesized by K. xylinus in previous studies, confirming the chemical and structural identity of the material synthesized in this study.

4. Purification Efficacy and Overall Assessment

The alkaline purification procedure that we used in this experiment, including the treatment with 0.5–1.0 M NaOH for 1–2 hours and several washes with distilled water until reaching a neutral pH (7.0), was extremely effective in eliminating bacterial cellular components and the rest of the culture medium constituents in the collected

pellicles. The SEM images, showing structurally intact nanofibrillar membranes with absent cellular debris and FTIR spectra, showing no extraneous absorption bands that could be due to the presence of proteinaceous or lipopolysaccharide contaminants all supported the success of this purification step. This is in accordance with the results of reference [6] which categorised that the alkaline treatment is both required and adequate to produce highly purified BC membranes to be used in further characterization and downstream applications according to reference [7]. All the gravimetric, morphological and spectroscopic data collected in the current work allow concluding that the locally isolated strain of K. xylinus can generate high-quality BC in cost-effective laboratory conditions. The physicochemical characteristics of the material obtained, such as the high WHC, the developed nanofibrillar structure, the high crystallinity, and chemical purity, make it a good candidate for environmental applications, especially in the adsorption of heavy metals and water treatment.

Conclusions

The findings in Table (1) showed that the wet-weight of BC pellicles (25.82–27.33 g) were significantly higher than the respective dry-weight (0.13-0.15 g). This is a very strong difference between hydrated and dehydrated mass that has been well reported as characteristic of bacterial cellulose and attributed to its unusually high water-holding capacity (WHC). The wet mass of BC is much higher than its dry mass because of the development of a three-dimensional, hydroxyl-rich nanofibrillar network [7]. The large percentage of water trapped in the fibrillar network under wet conditions explains the significantly high wet weight compared to dry weight recorded in the experiment conducted.

The reproducibility of BC production and the consistency of the fermentation process is further supported by the consistency in the wet and dry weights determinations in most of the replicates. Regular formation of pellicles along the air-liquid interface is conditional on maintained environmental conditions, especially, on the constant temperature and sufficient oxygen presence [7]. The small variation in wet weight values observed between the six replicates was thus due to the reproducible and consistent cultivation parameters used during the study.

The difference in wet-to-dry weight as is in this research is compatible with other studies carried out in the past that have confirmed the distinctive structural form of BC to store water in high concentration levels far above its dry weight [6]. Additionally, through the progressive and constant pellicle generation under conditions of stationary fermentation the suitability of the methodology of production as well as the effective organization of the locally isolated strain is also confirmed, which confirms the fact that the given methodology is suitable to produce high-quality BC under the conditions of the local laboratory.

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