polysaccharides

MDPI

Article

Bacterial Cellulose Purification with Non-Conventional, Biodegradable Surfactants

Alireza Nikbakht †, Elizabeth M. van Zyl †, Stephen Larson, Sawyer Fenlon and Jeannine M. Coburn *

Department of Biomedical Engineering, Worcester Polytechnic Institute, 100 Institute Road, Worcester, MA 01609, USA

* Correspondence: jmcoburn@wpi.edu; Tel.: +1-(508)-831-6839

† These authors contributed equally to this work.

Abstract: Bacterial cellulose (BC) is a versatile biopolymer with significant potential across biomedical, food, and industrial applications. To remove bacterial contaminants, such as protein and DNA, BC pellicles undergo purification, which traditionally relies on harsh alkali treatments, such as sodium hydroxide or strong surfactants, which present environmental concerns. In response, this study evaluates the efficacy of various non-conventional surfactants—both non-biodegradable and biodegradable—as alternatives for BC purification. Among the surfactants tested, sodium cocoyl isethionate (SCI), a mild anionic and biodegradable surfactant, emerged as particularly effective, achieving an 80.7% reduction in protein content and a 65.19% reduction in double-stranded DNA (dsDNA) content relative to untreated samples. However, these advantages were not without additional challenges, such as the appearance of residual surfactants. Given SCI's promising performance and biodegradability, it was further examined in two-step treatment protocols; additionally, sodium dodecyl sulfate (SDS) was also examined as a more traditional anionic surfactant as well as NaOH. For the two-step treatment protocol, BC pellicles were treated with one reagent for 3 h, followed by a second reagent for an additional 3 h. Notably, by using NaOH as the final step in the two-step treatment protocol, residual surfactant was not detected in the FTIR analysis. Overall, this work demonstrates that SCI, in addition to subsequent NaOH treatment, can be used as a surfactant-based approach for BC purification, representing a potential environmentally friendly alternative to traditional surfactant-based approaches for BC purification.

Keywords: bacterial cellulose; purification; non-conventional surfactants; biodegradable surfactants; non-biodegradable surfactants; sodium cocoyl isethionate

Citation: Nikbakht, A.; van Zyl, E.M.; Larson, S.; Fenlon, S.; Coburn, J.M. Bacterial Cellulose Purification with Non-Conventional, Biodegradable Surfactants. *Polysaccharides* **2024**, *5*, 857–871. https://doi.org/10.3390/polysaccharides5040053

Academic Editor: Artur Valente

Received: 27 September 2024
Revised: 4 November 2024
Accepted: 4 December 2024
Published: 9 December 2024

1. Introduction

In the field of biomedical engineering, advanced biomaterials are explored for many applications, including wound care. Cellulose, a natural polymer, stands out as a promising candidate owing to its intrinsic properties, including biocompatibility, water-holding and absorption capability, non-adhesiveness, gas permeability, and favorable mechanical characteristics, which make it an appealing choice for wound care [1–3]. Its abundance and ease of production further enhance its suitability as a biomaterial. Cellulose is composed of glucose units connected by β(1–4) glycosidic bonds and has been investigated as a biomaterial for bone tissue engineering, cartilage tissue engineering, wound dressing, engineering blood vessels, skin regeneration, and drug delivery applications [4–12].

Bacterial cellulose (BC), synthesized using *Komagataeibacter* spp. including *K. xylinus*, *K. hansenii*, *K. rhaeticus*, and *K. sucrofermentans*, is recognized for its high yield and purity, offering an advantageous alternative to plant-derived cellulose [13,14]. This shift towards bacterial sources for cellulose not only enhances the sustainability of biomaterials development but also circumvents the extensive processing required to extract cellulose from plants, thereby offering materials with higher mechanical strength and crystallinity

directly [15–18]. However, despite its inherent purity compared to other polymeric contaminants, BC requires thorough purification to eliminate any residual bacterial contaminants, such as DNA and proteins, to prevent immune reactions. The conventional purification strategy often involves the use of aqueous sodium hydroxide (NaOH) for alkali treatment and surfactants, necessitating subsequent neutralization and/or extensive washing to achieve a neutral pH and surfactant removal, respectively [18–20].

Surfactants, widely recognized for their detergent and emulsification capabilities, have emerged as vital in biomedical applications, particularly for the purification of biomaterials such as BC. These agents, divided into ionic (including cationic, anionic, and zwitterionic) and non-ionic categories, exhibit molecular structures with hydrophilic heads and hydrophobic tails, which help to solubilize and remove impurities [21,22]. Traditionally utilized purification methods, such as alkali treatment with NaOH [23,24], have been shown to effectively remove bacterial contaminants from BC. Amarasekara et al. demonstrated that NaOH treatment is efficient in purifying BC, particularly when combined with subsequent bleaching steps using hydrogen peroxide or sodium hypochlorite, which significantly improve the whiteness and purity of the cellulose. While NaOH is effective, synthetic surfactants, such as sodium dodecyl sulfate (SDS) and Triton X-100 (TX), are commonly employed in various purification processes due to their ability to disrupt cell membranes and solubilize impurities [25–27]. However, synthetic surfactants, commonly used in household cleaners [28,29], cosmetics [30], and pharmaceuticals [31,32], pose environmental challenges due to their non-biodegradability [33,34]. In contrast, biodegradable surfactants derived from natural sources, such as plant oils, offer eco-friendly alternatives, reducing environmental impact [35]. These agents have the potential to replace synthetic surfactants in various applications, including tissue decellularization and scaffold fabrication for tissue engineering [36,37].

Naturally derived, degradable, and biocompatible surfactants may be an alternative to traditional surfactants. Surfactants derived from natural sources, such as plants, microorganisms, and animals, have been shown to not accumulate within the environment, reducing the potential for environmental pollution and offering a green alternative to their synthetic counterparts [38]. Their ability to effectively reduce surface and interfacial tension, combined with their minimal ecological footprint, underscores their potential biomaterial purification.

In this work, we compare the use of traditional synthetic surfactants (SDS, Triton-X 100, and CHAPS) with three biodegradable surfactants, namely sodium cocoyl isethionate (SCI), dodecyl glucoside (DG), and cocamidopropyl betaine (CB), to determine if biodegradable surfactants are a viable alternative to traditional surfactants for BC purification. By evaluating the effectiveness of these surfactants in removing residual double-stranded DNA (dsDNA) and proteins, our study aims to advance a more sustainable method for preparing BC for biomedical applications. This effort seeks to establish a new paradigm in the biomaterials field, aligning with a growing emphasis on environmentally responsible research practices.

2. Materials and Methods

2.1. Materials

Komagataeibacter hansenii NQ5 (ATCC 53582) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Bacto™ peptone and Difco™ yeast extract were purchased from Becton Dickinson and Company (Franklin Lakes, NJ, USA). Agar was purchased from Sunrise Science Products (San Diego, CA, USA). Sodium chloride (ACS reagent, ≥99% purity) was purchased from Alfa Aesar (Ward Hill, MA, USA). D-glucose (BioReagent, ≥99.5% purity), citric acid (99% purity), sodium phosphate dibasic (BioReagent, ≥99.5% purity), sodium hydroxide (ACS reagent, ≥97.0% purity), calcium chloride (≥93% purity), ethylenediaminetetraacetic acid (EDTA), and Triton X-100 (TX) were purchased from Millipore Sigma (ST. Louis, MO, USA). Clear, flat-bottomed well plates were purchased from Greiner Bio-One (Monroe, NC, USA), and Falcon™ round-

bottom test tubes were purchased from Corning, Inc. (Corning, NY, USA). Sodium cocoyl isethionate (SCI) was sourced from Artekas Innovation® (Klaipėda, Lithuania). Sodium dodecyl sulfate (SDS) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from Avantor® (Radnor, PA, USA). Decyl glucoside (DG) was sourced from Cocojojo LLC (Santa Ana, CA, USA). Cocamidopropyl betaine (CB) was sourced from H&B Oils Center Co. (Westchester, IL, USA). Distilled water, L-cysteine, sodium hydrogen phosphate, and guanidine hydrochloride were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Papain was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Deionized water was produced via a PicoPure® Water Purification System (Hydro Service and Supplies, Durham, NC, USA).

2.2. Bacterial Cellulose Production

Cellulose-producing bacteria *Komagataeibacter hansenii* NQ5 was cultured on Hestrin–Schramm (HS) agar plates (comprising 20 mg/mL glucose, 5 mg/mL peptone, 5 mg/mL yeast extract, 1.15 mg/mL citric acid, 2.7 mg/mL disodium phosphate, and 15 mg/mL agar dissolved in distilled water). These plates were incubated at 30 °C for four days to facilitate the selective formation of colonies. Single colonies were then cultured in HS liquid medium, which had the same composition as the agar medium except for the absence of agar, in 15 mL conical tubes at 30 °C for four days to promote optimal bacterial growth. When experiments necessitated larger volumes of inoculum, 40 mL culture was produced from the 15 mL conical tube using 10% (v/v) inoculum. To produce BC pellicles, HS media was inoculated with 10% by volume inoculum. The inoculated HS media was then distributed into 96-well plates, 200 µL per well, and incubated under static conditions at 30 °C in a humidified incubator for seven days to allow for the formation of BC pellicles. Following BC production, the BC pellicles underwent a series of treatment protocols to removal bacterial contaminants. These steps were designed to purify the BC by removing non-cellulosic substances, thus ensuring the cellulose's purity and structural integrity for further analytical evaluation.

2.3. Bacterial Cellulose Treatment Protocols

In this study, we utilized two treatment protocols to remove contaminants from the BC samples (Figure 1). For the one-step treatment protocol (Figure 1A), the following six surfactant groups were evaluated (all dissolved in deionized water to 2% w/v): SCI, SDS, CHAPS, TX, DG, and CB. A water-only treatment and a 0.1 M NaOH treatment were used as controls (negative and positive, respectively). Sodium hydroxide is considered the positive control for these experiments as NaOH treatment is a routine method for removing contaminants from BC. BC pellicles were immersed in the respective surfactant solution or control and incubated at 60 °C in an oven for 3 h, following a previously established method [39,40]. Subsequently, the samples were rinsed four times with distilled water for 20 min per rinse. For the two-step treatment protocol (Figure 1B), the samples were divided into 12 groups. The surfactant and NaOH concentrations were the same as the one-step treatment protocol. The two-step treatment protocol utilized an initial 3 h incubation step, followed by a subsequent 3 h incubation step where different reagents were used during each step as follows: water → water, NaOH → NaOH, SCI → SCI, SDS → SDS, NaOH → SCI, NaOH → SDS, SCI→ NaOH, SDS → NaOH, NaOH → water, and water → NaOH. Subsequently, the samples were rinsed four times with distilled water for 20 min per rinse.

The processed BC pellicles and untreated control pellicles were frozen at −80 °C and lyophilized using a respective maximum absorbance ® Triad™ Freeze Dry System (Models 74,000 Series) overnight. The lyophilization process was carried out at a condenser temperature of −85 °C, shelf temperature of −20 °C, and vacuum pressure of 0.22 mBar. All lyophilized BC pellicles were stored at −20 °C until further analysis.

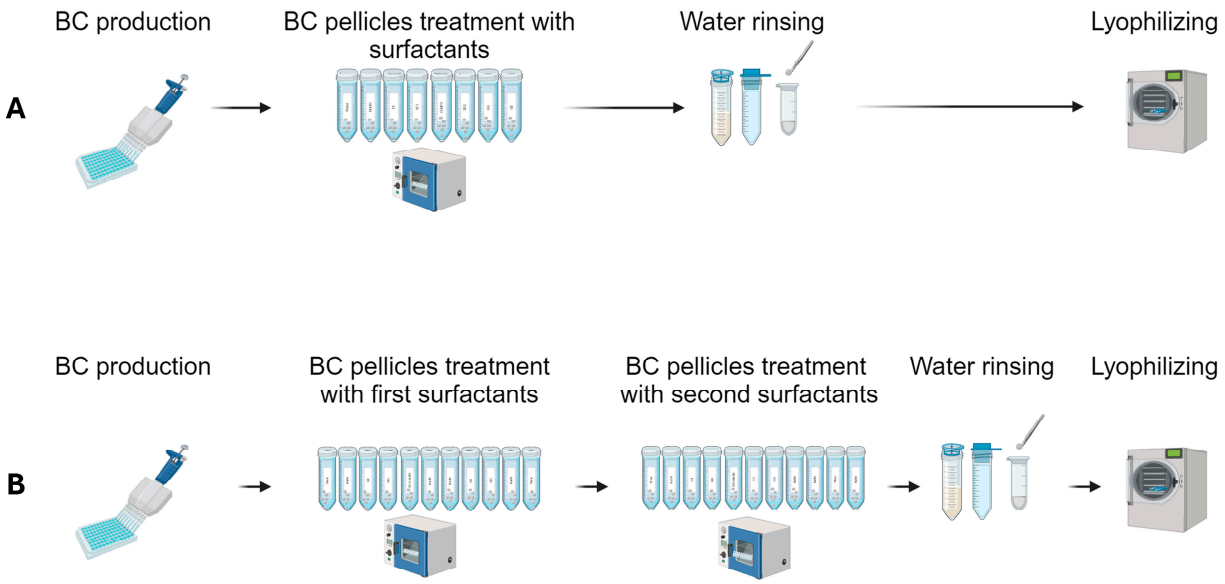


Figure 1. Schematic of the treatment protocols. (**A**) One-step treatment protocol and (**B**) two-step treatment protocol. In general, BC was produced over a 7-day culture period, then transferred to tubes to be treated with surfactants or various control conditions, followed by water rinsing and lyophilization. Created with BioRender.com.

2.4. Residual Protein Quantification

Protein in BC pellicles was quantified using a BCA Protein Assay Kit (Pierce™, Thermo Fisher Scientific), following the manufacturer's protocol with some modifications. For protein solubilization, BC pellicles were placed in 6 M guanidine hydrochloride at room temperature for 16 h followed by centrifugation at 16,000× *g* (14,000 RPM) for 6 min. The supernatant, in triplicate, and bovine serum albumin standard (0–2000 µg/mL), in duplicate (25 µL each), were loaded into clear, flat-bottom 96-well plates (Greiner Bio-One, Monroe, NC, USA). An equal volume of the BCA solution was added to each well, and the plate was incubated at 37 °C for 30 min. The solution absorbance at 562 nm was measured (SpectraMax M2, Molecular Devices, San Jose, CA, USA), and a standard curve was generated from the BSA solutions. The sample absorbance values were converted to protein concentrations (µg/mL) using the slope generated for the BSA standard curve.

2.5. Residual DNA Quantification

Residual dsDNA in the BC pellicles was quantified using a Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen™, Thermo Fisher Scientific), following the manufacturer's protocol with some modifications. BC pellicles were treated with papain solution (125 µg/mL papain, 11.1 mM L-cysteine, and 10 mM EDTA in PBS) at 60 °C for 16 h to extract residual DNA. The supernatant, in triplicate, along with the DNA standard (0–2 µg/mL), in duplicate (50 µL each), were loaded into black, flat-bottom 96-well plates. An equal volume of PicoGreen™ reagent (1:200 dilution in 1X Tris EDTA buffer) was added to each well, and the plate was incubated at room temperature for 5 min. The fluorescence excitation at 480 nm and emission at 520 nm were measured using a SpectraMax M2 plate reader. A standard curve was generated using lambda DNA, and the sample fluorescence values were converted to dsDNA concentrations (µg/mL) using the slope of the lambda DNA standard curve.

2.6. Fourier-Transform Infrared Spectroscopy Analysis

Fourier-transform infrared spectroscopy (FTIR) analysis was conducted using a Bruker's VERTEX 70v FT-IR Spectrometer (Bruker Corporation, Billerica, MA, USA). Spectra were acquired at a resolution of 4 cm^{-1} within a spectral range of 4500–600 cm^{-1}, and 32 scans av-

eraged for each measurement (data analysis focused on the range of 600 cm^{-1} to 3500 cm^{-1}). For data presentation, data underwent normalization at the maximum peak in each sample (1056.87 cm^{-1} for the untreated sample, 1032.72 cm^{-1} for the rest of the groups), and baseline adjustments via Python. All FTIR spectra were baseline-corrected by subtracting the minimum absorbance value from each spectrum. The spectra, within the wavenumber range reported in each plot, were then normalized to their respective maximum absorbance values to standardize intensity for comparison.

2.7. X-Ray Diffraction

X-ray diffraction (XRD) data were collected using a Rigaku MiniFlex 6G X-ray diffractometer (Rigaku Corporation, Tokyo, Japan). The system operates with a 600 W copper tube at a voltage of 40 kV and a current of 15 mA, using Cu Kα radiation with a wavelength of 0.154 nm. Data acquisition was conducted using SmartLab Studio II software (v4.6.355.0 Rigaku Corporation). Raw data processing included asymmetric least squares (ALS) baseline correction to remove background noise, followed by analysis using Python (v. 3.11.5).

2.8. Statistical Analysis

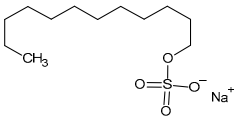
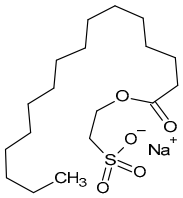
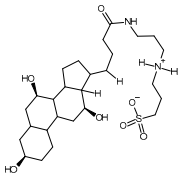
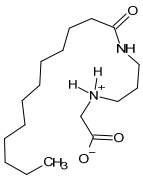
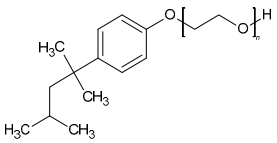
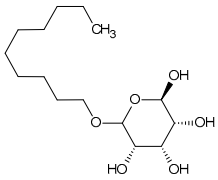
All statistical analyses were performed using GraphPad Prism 7, and visualization was conducted using Python. Statistical analysis of the effects of treatment agents on residual protein and dsDNA content was conducted using a one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test as specified in the figure caption. All data are presented as mean ± standard error of the mean. In this study, square root transformation was applied to stabilize variance and improve the normality of protein and dsDNA concentration data, aligning with established practices in biomedical research. Osborne [41] and Wallenstein et al. [36] emphasize the utility of transformations like square root for handling non-normally distributed data and ensuring robust statistical analyses.

3. Results and Discussion

3.1. Overview of Surfactant Selection

In the biomedical field, surfactants are utilized in tissue decellularization processes and are crucial for fabricating biocompatible scaffolds for tissue engineering applications [37,42]. The shift towards eco-friendly surfactants aligns with growing environmental concerns and the need for sustainable solutions in various industries, including biomedical research and development. In this work, various surfactants were evaluated for removing bacterial cell contaminants from BC samples, considering both their cleaning efficacy and eco-friendliness (Table 1). Surfactants were categorized into two groups: eco-friendly and non-eco-friendly. Among the eco-friendly options were SCI, derived from coconut oil [43], CB, also sourced from coconut oil [44], and DG, boasting natural derivation and biodegradability [45]. SCI, CB, and DG are recognized for their mild and skin-friendly properties, making them suitable for applications where products come into contact with delicate tissues, such as personal care products [46–52]. In contrast, there are less-eco-friendly options, like SDS, which is commonly used and known for its strong detergent properties [53–55]. It is derived from both natural and synthetic sources, and its environmental impact is a significant concern [56]. Triton-X is widely applied in biological research, and its derivation from petroleum makes it less sustainable compared to the other surfactants [57–59]. CHAPS, known for its mildness and often employed in biochemical and biotechnological applications for protein solubilization [60,61], is effective in cleaning but poses environmental concerns due to its synthetic origins and limited biodegradability. This information guided our selection process, wherein we prioritized surfactants aligned with both cleaning efficacy and environmental sustainability principles.

Table 1. Surfactant list with associated chemical structure, surfactant category, and degradation information.

Surfactants	Chemical Structure	Category	Degradability	References
Sodium Dodecyl Sulfate (SDS)		Anionic	Non-degradable or degradable by bacteria	[53–55]
Sodium Cocoyl Isethionate (SCI)		Anionic	Hydrolytically degradable ester bond	[46,47]
CHAPS		Zwitterionic	Non-degradable	[60,61]
Cocamidopropyl Betaine (CB)		Zwitterionic	Hydrolytically degradable amide bond	[48–51]
Triton-X 100 (TX)		Non-ionic	Non-degradable	[57,58]
Decyl Glucoside (DG)		Non-ionic	Biodegradable	[52]

3.2. Protein and DNA Removal Using the One-Step Treatment Protocol

To explore the ability of the newly evaluated surfactants to remove protein and DNA contaminants, a single-treatment-step protocol was explored. The protocol was based on established methods for removing contaminants from BC [39,40], extending these to include the additional surfactants explored in this work. Residual protein content normalized to the untreated control group showed that NaOH and SDS were effective at removing protein contaminants, reducing them to 3% and 5.6% of the initial protein amount, respectively (Figure 2A). Water treatment alone reduced the residual protein content, indicating a baseline ability to remove proteins. When comparing them to protein removal with water alone, NaOH and SDS removed 84.6% and 71.6% more protein, respectively. SCI also demonstrated a significant impact, reducing protein contaminants to 16.2% of the initial protein amount, which was similar to that of the water-only treatment. Other surfactants, such as TX, CHAPS, DG, and CB, exhibited similar protein contents compared to water treatment, suggesting that zwitterionic, non-ionic surfactants, and mild anionic surfactants are not effective at protein removal on their own. When assessing residual DNA content for the one-step treatment protocol (Figure 2B), NaOH emerged as highly effective, achieving a 97.8% reduction in dsDNA content. SDS and SCI also demonstrated significant dsDNA re-

moval, with reductions of 87.7% and 86.1% compared to the untreated sample, respectively. These results guided the surfactant selection for the two-step treatment protocol.

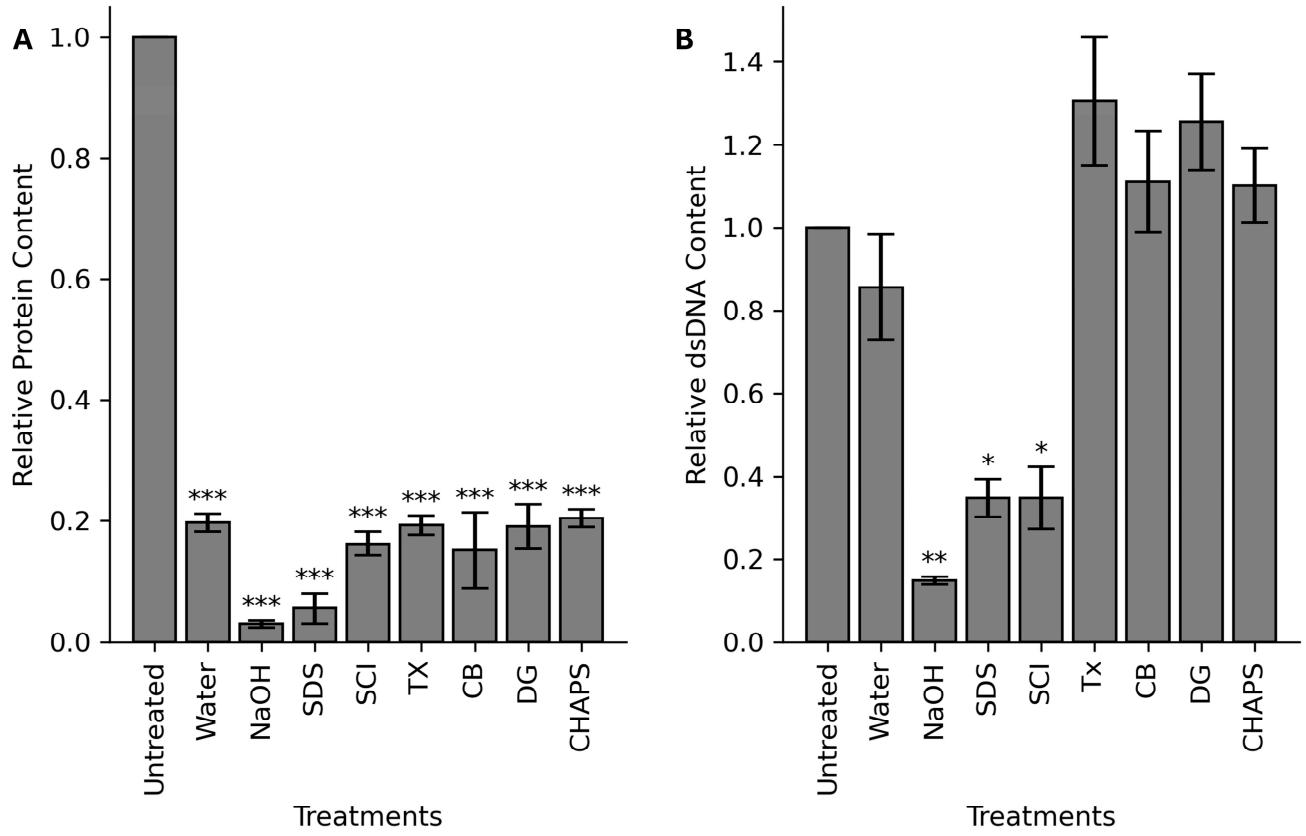


Figure 2. Quantification of relative protein and dsDNA content in BC pellicles after undergoing the one-step treatment protocol. (**A**) Relative protein content of BC pellicles after treatment, reported as normalized to the untreated control group. (**B**) Relative dsDNA content of BC pellicles after treatment, reported as normalized to the untreated control group. Data are presented as the mean ± SEM from three independent experiments (N = 3), with three independent samples (n = 3) per experiment. For statistical analysis, data were square root transformed and then normalized. Statistical differences compared to the untreated control group were determined using ANOVA followed by Tukey's HSD test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3. FTIR Analysis to Evaluate Contaminate Removal from BC Pellicles Via the One-Step Treatment Protocol

In the FTIR spectra analysis of BC pellicles that underwent the one-step treatment protocol, several distinct peaks were observed (Figure 3A), each correlating with specific groups within the cellulose structure, affirming its molecular composition. Peaks in the range of 1030 to 1100 cm^{-1} (1031 and 1056 cm^{-1}) were attributed to C-O-C stretching vibrations, indicative of ether linkages in the cellulose structure (Figure 3B). The 1620 to 1640 cm^{-1} range was indicative of amide bonds, while the 1630 to 1650 cm^{-1} range may have corresponded to O-H bending vibrations. The reduced intensity of the peak within this general region in the samples treated with surfactants, compared to the untreated sample, suggests that these treatments were more effective in removing protein contaminants as indicated by the diminished amide bond peak (Figure 3C) [62]. Small peaks between 1750 and 1700 cm^{-1} were also detected in SCI-treated samples, which likely correspond to carbonyl (C=O) stretching of ester groups from residual SCI [63–65]. A broad peak around 3342 cm^{-1}, associated with O-H stretching vibrations, was observed in the untreated sample. This peak became sharper after treatment, suggesting that all the treatment groups, even the water-only treatment, removed water-soluble O-H containing impurities

(Figure 3D). In all the treatment groups, the broad peak observed in the 3000 cm^{-1} to 2800 cm^{-1} range could be attributed to C-C stretching of CH_2 and CH_3 groups or CH_2 asymmetric stretching of BC. Furthermore, peaks associated with C-H stretching vibrations were observed at approximately 2852 cm^{-1} and 2920 cm^{-1} in the SCI-, CB-, and DG-treated samples, suggesting that residual surfactant was present within these samples (Figure 3D). Notably, analysis of the two-step treatment protocol provided a means to remove residual SCI as reported in subsequent results.

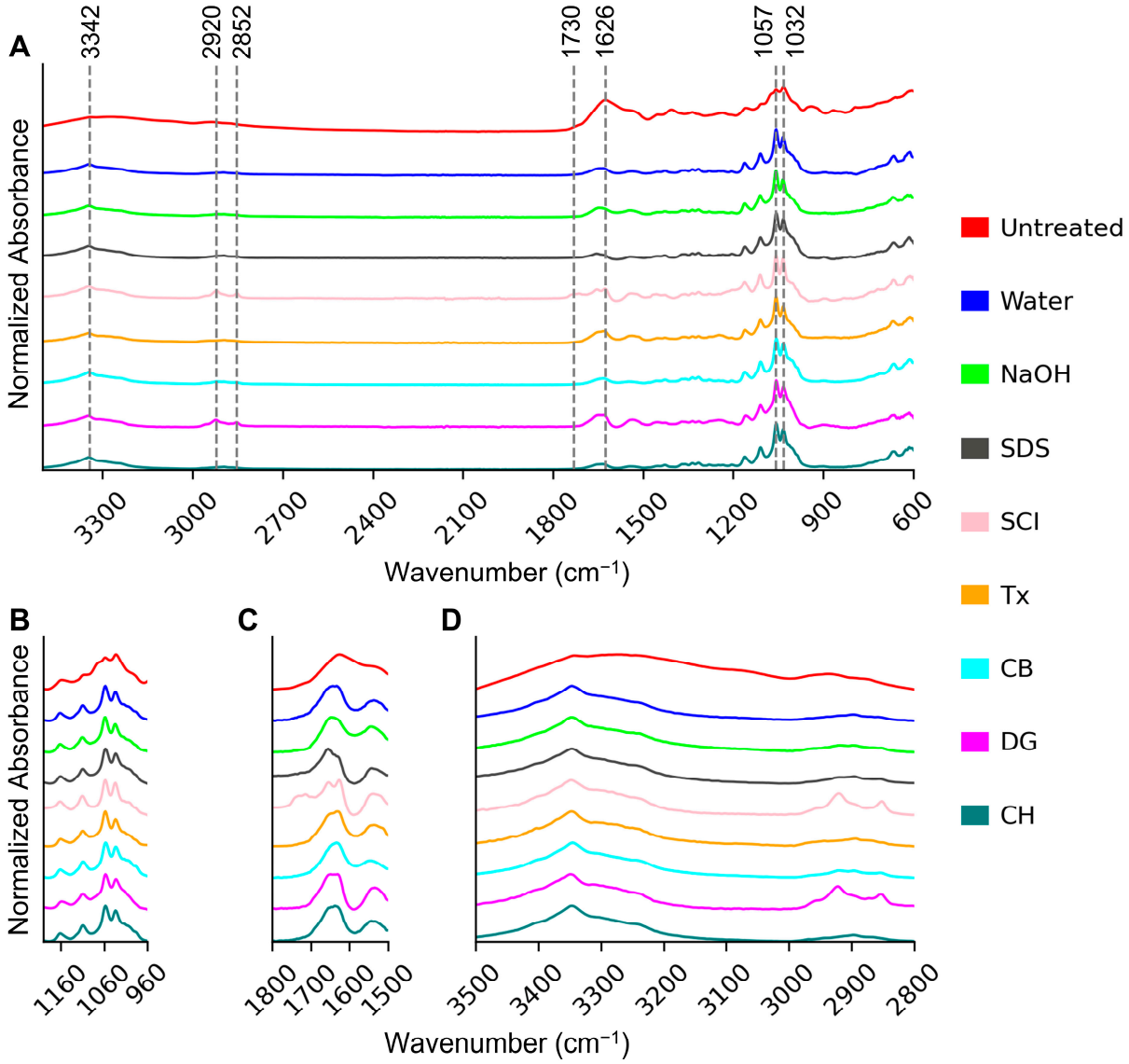


Figure 3. FTIR analysis of BC pellicles after treatment with the one-step treatment protocols or from untreated samples. (**A**) FTIR spectra for BC pellicles with specified regions associated with (**B**) C-O stretching, (**C**) amide bond and O-H bending, and (**D**) C-C stretching or CH_2 asymmetric stretching in BC and C-H stretching in residual surfactant.

3.4. The Two-Step Treatment Protocol Shows an Improvement in the Removal of Protein and DNA Using Degradable Anionic Surfactant

To improve protein removal from BC, a two-step treatment protocol was developed, leveraging insights from the most effective surfactants identified in the one-step treatment procedure. This subsequent protocol incorporated a sequential surfactant or NaOH application to facilitate improved sample purification. In this approach, the no-treatment control continued to show the highest levels of proteins, serving as a baseline for comparison

(Figure 4A). All treatments, including the water-only treatment, as observed in the one-step protocol, reduced protein content. Residual protein was virtually undetectable with NaOH → NaOH and NaOH → SCI. These findings suggest that NaOH is an important reagent for protein removal. When evaluating residual DNA quantity for the samples processed with the two-step treatment protocol (Figure 4B), the water-only treatment resulted in a 26% decrease in dsDNA content, indicating a moderate though inadequate ability to remove DNA. The SCI → SCI treatment demonstrated strong effectiveness with an 85% decrease, while SDS → SDS achieved an 80% decrease. The NaOH → SCI and NaOH → SDS treatments achieved 81% and 82% decreases, respectively. Notably, the sequential treatments of SCI → NaOH and SDS → NaOH exhibited the highest reductions in dsDNA content, with decreases of 93% and 92%, respectively.

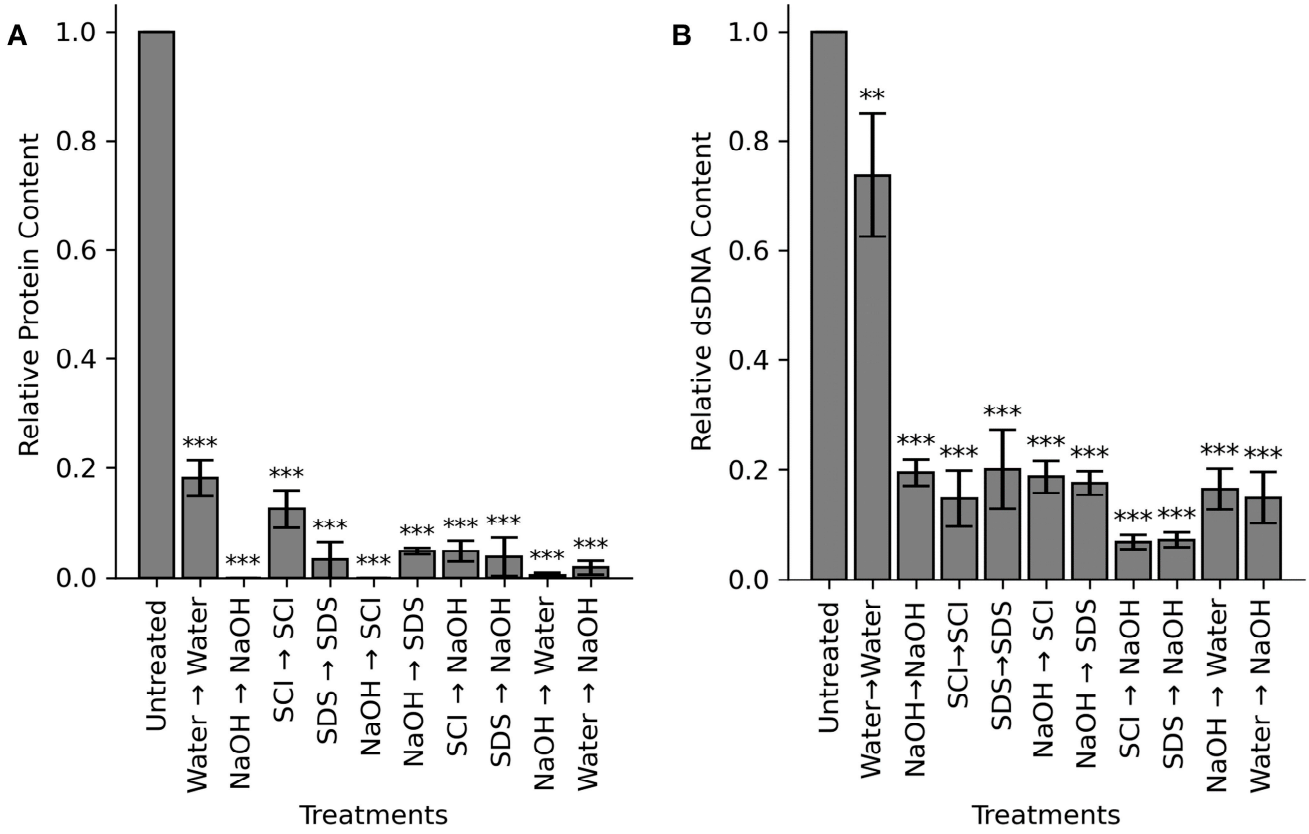


Figure 4. Quantification of relative protein and dsDNA in BC pellicles after undergoing the two-step treatment protocol. (**A**) Relative protein content of BC pellicles after treatment reported as normalized to the untreated control group. (**B**) Relative dsDNA content of BC pellicles after treatment reported as normalized to the untreated control group. Data are presented as the mean ± SEM from three independent experiments (N = 3), with three independent samples (n = 3) per experiment. For statistical analysis, the data were square root transformed and then normalized. Statistical differences compared to the untreated control group were determined using ANOVA followed by Tukey's HSD test (** $p < 0.01$, *** $p < 0.001$).

3.5. FTIR Analysis Confirms Improved Contaminant Removal, and Residue Surfactant Removal Using the Two-Step Treatment Protocol

In the investigation of a two-step treatment protocol utilizing SDS and SCI as selected surfactants, FTIR analyses were performed on treated BC samples (Figure 5A). As expected, peaks in the range of 1030 to 1100 cm^{-1} (1033 and 1057 cm^{-1}), attributed to C-O-C stretching vibrations, were observed, indicative of ether linkages in the cellulose structure (Figure 5B). The two-step treatment protocol samples, except for the untreated sample, did not show a significant peak at 1625 cm^{-1}, which was indicative of amide

groups (Figure 5C). This, along with a qualitative comparison of the relative protein content results (Figures 2 and 4), indicated that the two-step treatment protocol was more effective in removing protein from the BC pellicles. Furthermore, SCI → NaOH treatment was effective at removing residual SCI. Specifically, peaks associated with SCI contaminate were observed at 2918 cm^{-1} and 2850 cm^{-1}, and small peaks between 1750 and 1700 cm^{-1} in the SCI → SCI and NaOH → SCI groups, while the SCI → NaOH group did not exhibit these peaks (Figure 5D). Concurrently, the peaks at 1033 cm^{-1}, 1056 cm^{-1}, 2937 cm^{-1}, and 3342 cm^{-1} remained consistent across treatments, indicating the BC structure was well-preserved. These characteristic cellulose peaks collectively affirm the molecular integrity of BC after the two-step treatment protocols.

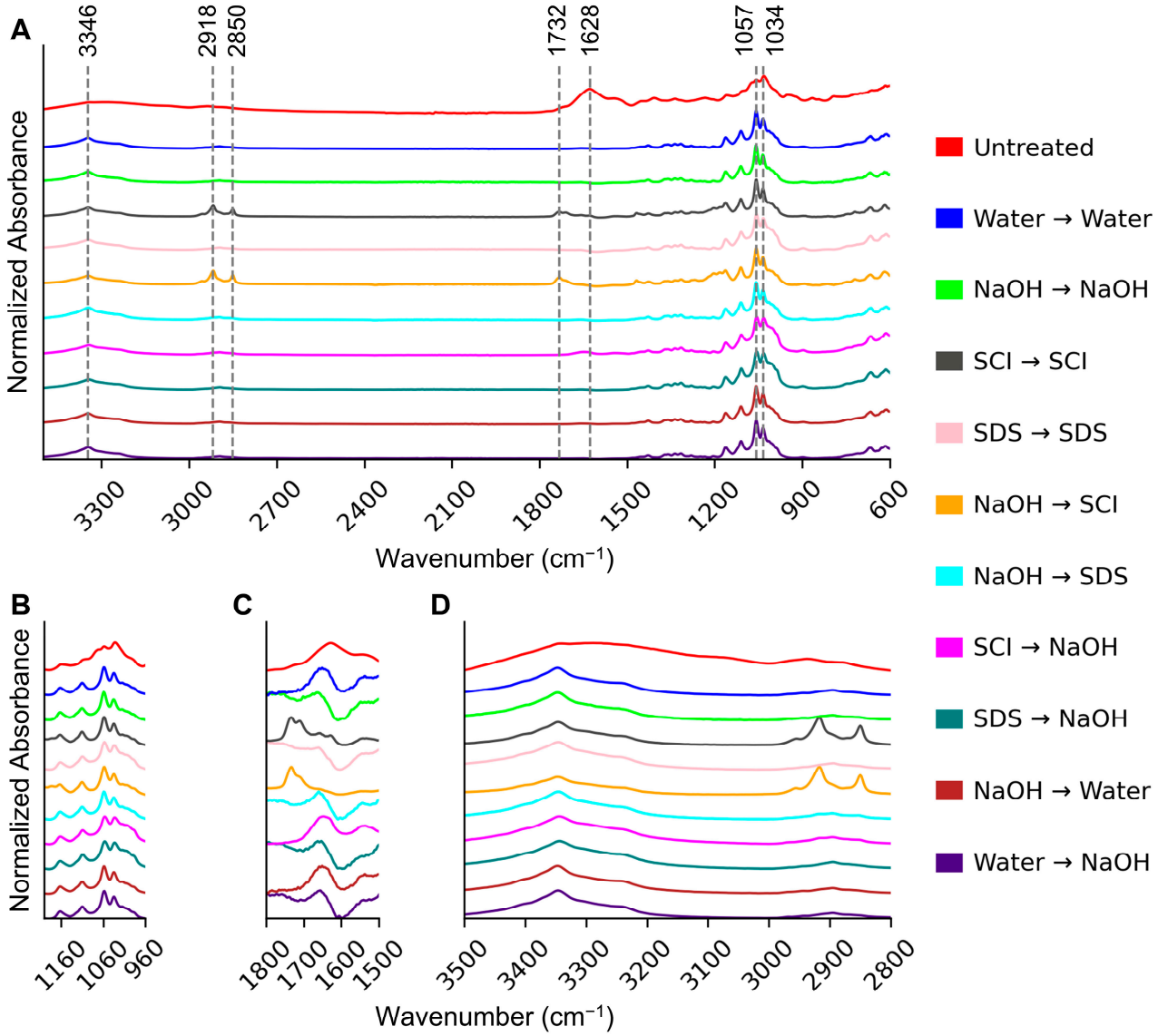


Figure 5. FTIR analysis of BC pellicles after treatment with the two-step treatment protocols or from untreated samples. (**A**) FTIR spectra for BC pellicles with specified regions associated with (**B**) C-O stretching, (**C**) amide bond and O-H bending, and (**D**) C-C stretching or CH_2 asymmetric stretching in BC and C-H stretching in residual surfactant.

In addition to the FTIR analysis confirming improved contaminant removal, there was less residual surfactant contamination using the two-step treatment protocol. SCI, followed by NaOH, not only showed the highest efficacy in reducing protein content by 95.32% and dsDNA content by 93.25% but also demonstrated surfactant removal (Figure 5D).

This indicates that SCI can be used as a biodegradable surfactant, in series with NaOH, to achieve protein and dsDNA removal.

3.6. XRD Analysis Confirms Maintenance of Characteristic BC Crystalline Structure

To further characterize the BC pellicles that underwent the two-step treatment protocol, XRD was performed to analyze the crystalline structure (Figure 6). Asymmetric least square baseline correction was implemented prior to XRD evaluation [66,67]. The XRD pattern of untreated bacterial cellulose demonstrated typical features of native cellulose, including the presence of both crystalline and amorphous regions. Despite the diversity in chemical treatments, the XRD patterns consistently displayed three principal peaks at approximate 2θ values of 14.2°, 16.6°, and 22.4°. These three peaks are characteristic of the (100) plane, (010) plane, and (110) plane, respectively, of cellulose Iα, or the (1–10) plane, (110) plane, and 200 (plane), respectively, of cellulose Iβ [68–70]. This uniformity suggests that the structural integrity of the cellulose's crystallinity remained largely unaffected by the different treatment protocols. The observed broad peak at approximately 18.3° in untreated BC suggests a higher content of amorphous material or contaminants within the BC structure. Notably, this peak was absent in samples that underwent any of the two-step treatment protocols. Considering this, in addition to protein quantification, dsDNA quantification, and FTIR results, the broad peak was most likely attributed to bacterial contaminants within the untreated BC pellicles.

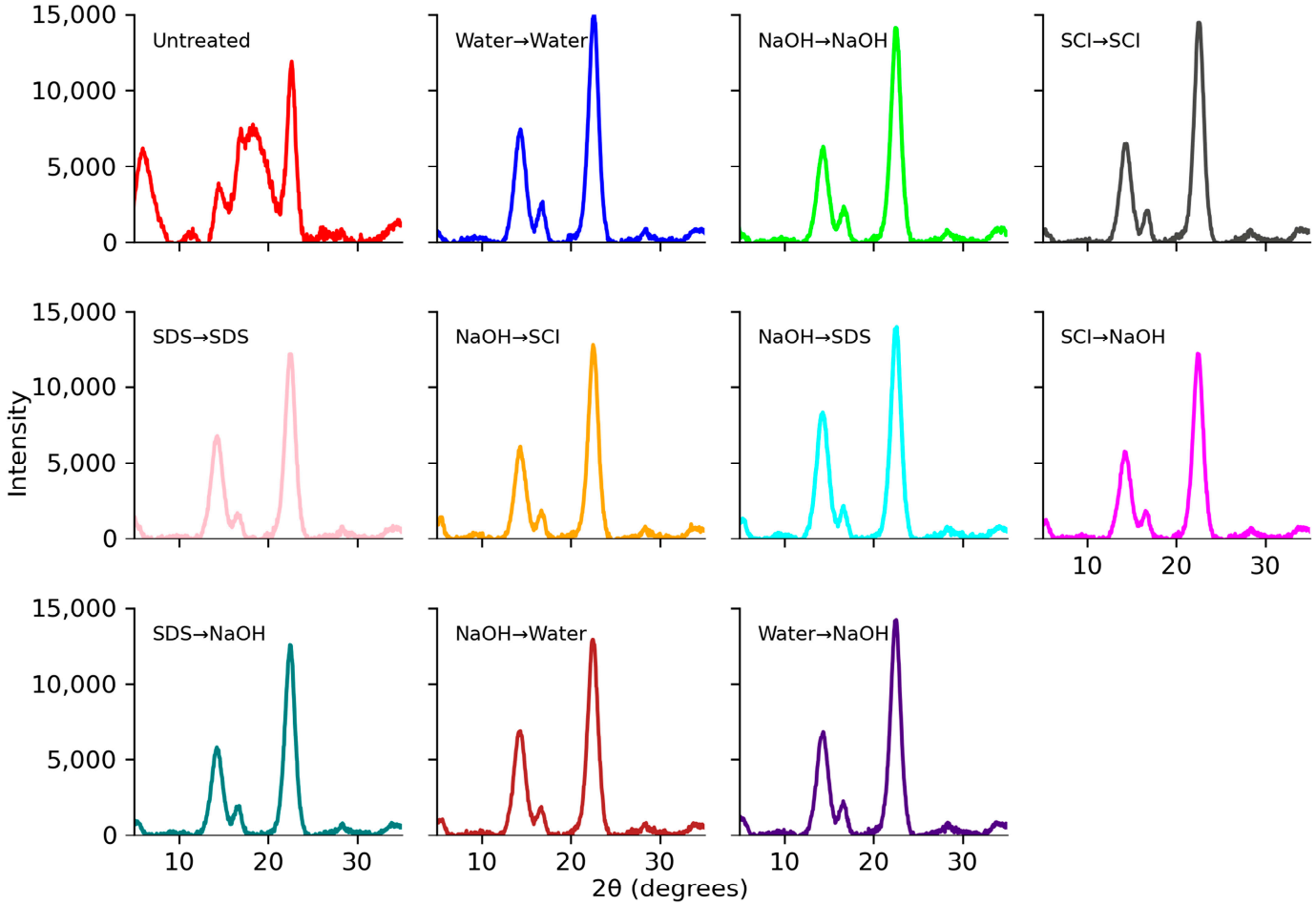


Figure 6. XRD diffraction patterns of BC pellicles left untreated or subjected to two-step treatment protocols. No obvious differences in the XRD diffraction pattern were observed across any of the two-step treatment protocols.

4. Conclusions

This study evaluated various surfactants and treatment protocols for BC purification, taking an environmentally friendly viewpoint. In the one-step treatment protocol, NaOH and SDS demonstrated the highest efficacy in dsDNA and protein removal. SCI also performed well compared to the other surfactants evaluated, suggesting that the anionic surfactant class is superior to other types of surfactants in contaminant removal. However, some residual SCI remained within the BC pellicles, and it was postulated that a multi-step process could be designed to remove the residual SCI while further removing bacterial contaminants. The results showed that the SCI → NaOH treatment performed similarly to SDS → NaOH in terms of protein and dsDNA removal. Interestingly, NaOH → SCI resulted in a lower residual protein content than SCI → NaOH, although there was the challenge of residual surfactant, which was not detected with the SCI → NaOH treatment. Importantly, XRD analysis confirmed that the structural integrity of BC was preserved across all treatments, including those involving SCI and NaOH. Taken together, the SCI → NaOH protocol emerged as the optimal approach, offering a balance between the use of a mild, biodegradable surfactant, effective contaminant removal, and undetectable residual surfactant based on the FTIR results.

Author Contributions: Conceptualization, E.M.v.Z. and J.M.C.; methodology, A.N., E.M.v.Z., S.L. and J.M.C.; formal analysis, A.N., E.M.v.Z., S.L., S.F. and J.M.C.; investigation, A.N., E.M.v.Z., S.L. and S.F.; data curation, A.N. and J.M.C.; writing—original draft preparation, A.N. and J.M.C.; writing—review and editing, A.N., E.M.v.Z., S.L., S.F. and J.M.C.; visualization, A.N. and J.M.C.; supervision, J.M.C.; funding acquisition, J.M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported, in part, by the National Science Foundation CAREER award DMR-2236940 to JMC.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated and/or analyzed during the current study are included in this published article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Helenius, G.; Bäckdahl, H.; Bodin, A.; Nannmark, U.; Gatenholm, P.; Risberg, B. In vivo biocompatibility of bacterial cellulose. *J. Biomed. Mater. Res. Part A* **2006**, *76A*, 431–438. [CrossRef] [PubMed]
2. Esa, F.; Tasirin, S.M.; Rahman, N.A. Overview of Bacterial Cellulose Production and Application. *Agric. Agric. Sci. Procedia* **2014**, *2*, 113–119. [CrossRef]
3. Mazhar, U.-I.; Taous, K.; Joong Kon, P. Water holding and release properties of bacterial cellulose obtained by in situ and ex situ modification. *Carbohydr. Polym.* **2012**, *88*, 596–603. [CrossRef]
4. Chen, C.; Xi, Y.; Weng, Y. Recent Advances in Cellulose-Based Hydrogels for Tissue Engineering Applications. *Polymers* **2022**, *14*, 3335. [CrossRef]
5. Mujuan, P.; Yinghong, H.; Fansu, M.; Yong, Z.; Hui, L.; Manling, D.; Qianqian, M.; Qi, W.; Zhen, C.; Lianyu, C.; et al. Application of bacterial cellulose in skin and bone tissue engineering. *Eur. Polym. J.* **2020**, *122*, 109365. [CrossRef]
6. Lin, L.; Jiang, S.; Yang, J.; Qiu, J.; Jiao, X.; Yue, X.; Ke, X.; Yang, G.; Zhang, L. Application of 3D-bioprinted nanocellulose and cellulose derivative-based bio-inks in bone and cartilage tissue engineering. *Int. J. Bioprint.* **2023**, *9*, 637. [CrossRef] [PubMed]
7. Madhavi Latha, C.; Aditya, V.; Ninian Prem Prashanth, P.; Swati, D.; Sreenivasa Rao, P. Assessment of properties, applications and limitations of scaffolds based on cellulose and its derivatives for cartilage tissue engineering: A review. *Int. J. Biol. Macromol.* **2021**, *175*, 495–515. [CrossRef]
8. Tudoroiu, E.-E.; Dinu-Pîrvu, C.-E.; Albu Kaya, M.G.; Popa, L.; Anuța, V.; Prisada, R.M.; Ghica, M.V. An Overview of Cellulose Derivatives-Based Dressings for Wound-Healing Management. *Pharmaceuticals* **2021**, *14*, 1215. [CrossRef]
9. Das, M.; Zandraa, O.; Mudenur, C.; Saha, N.; Sáha, P.; Mandal, B.; Katiyar, V. Composite Scaffolds Based on Bacterial Cellulose for Wound Dressing Application. *ACS Appl. Bio Mater.* **2022**, *5*, 3722–3733. [CrossRef]
10. Shanshan, Z.; Ran, Z.; Hua, C.; Yudong, L.; Jianhai, Z.; Xiao, C.; Guixing, Q.; Zhihong, W.; Guang, Y. Investigation on artificial blood vessels prepared from bacterial cellulose. *Mater. Sci. Eng. C* **2015**, *46*, 111–117. [CrossRef]

11. Maximilian, S.; Stefanie, R.; Dieter, K.; Anja, S.-K.; Maria, G.; Thomas, R.; Georg, L.; Navid, M.; Thorsten, W.; Jens, W. In vivo application of tissue-engineered blood vessels of bacterial cellulose as small arterial substitutes: Proof of concept? *J. Surg. Res.* **2014**, *189*, 340–347. [CrossRef]
12. Pajorova, J.; Skogberg, A.; Hadraba, D.; Broz, A.; Travnickova, M.; Zikmundova, M.; Honkanen, M.; Hannula, M.; Lahtinen, P.; Tomkova, M.; et al. Cellulose Mesh with Charged Nanocellulose Coatings as a Promising Carrier of Skin and Stem Cells for Regenerative Applications. *Biomacromolecules* **2020**, *21*, 4857–4870. [CrossRef]
13. Kaczmarek, M.; Jędrzejczak-Krzepkowska, M.; Ludwicka, K. Comparative Analysis of Bacterial Cellulose Membranes Synthesized by Chosen Komagataeibacter Strains and Their Application Potential. *Int. J. Mol. Sci.* **2022**, *23*, 3391. [CrossRef] [PubMed]
14. Brugnoli, M.; Robotti, F.; La China, S.; Anguluri, K.; Haghighi, H.; Bottan, S.; Ferrari, A.; Gullo, M. Assessing effectiveness of Komagataeibacter strains for producing surface-microstructured cellulose via guided assembly-based biolithography. *Sci. Rep.* **2021**, *11*, 19311. [CrossRef]
15. Ahmed, J.; Gultekinoglu, M.; Edirisinghe, M. Bacterial cellulose micro-nano fibres for wound healing applications. *Biotechnol. Adv.* **2020**, *41*, 107549. [CrossRef] [PubMed]
16. Huang, Y.; Zhu, C.; Yang, J.; Nie, Y.; Chen, C.; Sun, D. Recent advances in bacterial cellulose. *Cellulose* **2014**, *21*, 1–30. [CrossRef]
17. de Amorim, J.D.P.; da Silva Junior, C.J.G.; de Medeiros, A.D.L.M.; do Nascimento, H.A.; Sarubbo, M.; de Medeiros, T.P.M.; Costa, A.F.d.S.; Sarubbo, L.A. Bacterial Cellulose as a Versatile Biomaterial for Wound Dressing Application. *Molecules* **2022**, *27*, 5580. [CrossRef]
18. Santos, S.M.; Carbajo, J.M.; Quintana, E.; Ibarra, D.; Gomez, N.; Ladero, M.; Eugenio, M.E.; Villar, J.C. Characterization of purified bacterial cellulose focused on its use on paper restoration. *Carbohydr. Polym.* **2015**, *116*, 173–181. [CrossRef]
19. Gea, S.; Reynolds, C.T.; Roohpour, N.; Wirjosentono, B.; Soykeabkaew, N.; Bilotti, E.; Peijs, T. Investigation into the structural, morphological, mechanical and thermal behaviour of bacterial cellulose after a two-step purification process. *Bioresour. Technol.* **2011**, *102*, 9105–9110. [CrossRef]
20. Tang, W.; Jia, S.; Jia, Y.; Yang, H. The influence of fermentation conditions and post-treatment methods on porosity of bacterial cellulose membrane. *World J. Microbiol. Biotechnol.* **2010**, *26*, 125–131. [CrossRef]
21. Muhammad, M.T.; Khan, M.N. Eco-friendly, biodegradable natural surfactant (Acacia Concinna): An alternative to the synthetic surfactants. *J. Clean. Prod.* **2018**, *188*, 678–685. [CrossRef]
22. De, S.; Malik, S.; Ghosh, A.; Saha, R.; Saha, B. A review on natural surfactants. *RSC Adv.* **2015**, *5*, 65757–65767. [CrossRef]
23. Han, J.; Shim, E.; Kim, H.R. Effects of cultivation, washing, and bleaching conditions on bacterial cellulose fabric production. *Text. Res. J.* **2019**, *89*, 1094–1104. [CrossRef]
24. Martínez Ávila, H.; Schwarz, S.; Feldmann, E.-M.; Mantas, A.; von Bomhard, A.; Gatenholm, P.; Rotter, N. Biocompatibility evaluation of densified bacterial nanocellulose hydrogel as an implant material for auricular cartilage regeneration. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 7423–7435. [CrossRef] [PubMed]
25. Juan-Colás, J.; Dresser, L.; Morris, K.; Lagadou, H.; Ward, R.H.; Burns, A.; Tear, S.; Johnson, S.; Leake, M.C.; Quinn, S.D. The mechanism of vesicle solubilization by the detergent sodium dodecyl sulfate. *Langmuir* **2020**, *36*, 11499–11507. [CrossRef]
26. Jones, M. Surfactants in membrane solubilisation. *Int. J. Pharm.* **1999**, *177*, 137–159. [CrossRef]
27. Miozzari, G.; Niederberger, P.; Hu, R. Permeabilization of microorganisms by Triton X-100. *Anal. Biochem.* **1978**, *90*, 220–233. [CrossRef]
28. Costa, M.F.; de Oliveira, A.M.; de Oliveira Junior, E.N. Biodegradation of linear alkylbenzene sulfonate (LAS) by Penicillium chrysogenum. *Bioresour. Technol. Rep.* **2020**, *9*, 100363. [CrossRef]
29. Corada-Fernández, C.; González-Mazo, E.; Lara-Martín, P.A. Evaluation of the anaerobic biodegradation of linear alkylbenzene sulfonates (LAS) using OECD 308 water/sediment systems. *J. Hazard. Mater.* **2018**, *360*, 24–31. [CrossRef]
30. Cserháti, T.; Forgács, E.; Oros, G. Biological activity and environmental impact of anionic surfactants. *Environ. Int.* **2002**, *28*, 337–348. [CrossRef]
31. Seedher, N. In vitro study of the mechanism of interaction of trifluoperazine dihydrochloride with bovine serum albumin. *Indian J. Pharm. Sci.* **2000**, *62*, 16–20.
32. Yushmanov, V.E.; Perussi, J.R.; Imasato, H.; Tabak, M. Interaction of papaverine with micelles of surfactants with different charge studied by 1H-NMR. *Biochim. Biophys. Acta (BBA)-Biomembr.* **1994**, *1189*, 74–80. [CrossRef]
33. Ivanković, T.; Hrenović, J. Surfactants in the environment. *Arh. Za Hig. Rada I Toksikol.* **2010**, *61*, 95–109. [CrossRef]
34. Peters, R.; Montemagno, C.; Shem, L.; Lewis, B. *Surfactant Screening of Diesel-Contaminated Soil*; Argonne National Lab: Lemont, IL, USA, 1990.
35. Pradhan, A.; Bhattacharyya, A. Quest for an eco-friendly alternative surfactant: Surface and foam characteristics of natural surfactants. *J. Clean. Prod.* **2017**, *150*, 127–134. [CrossRef]
36. Wallenstein, S.; Zucker, C.L.; Fleiss, J.L. Some statistical methods useful in circulation research. *Circ. Res.* **1980**, *47*, 1–9. [CrossRef]
37. Balestrini, J.L.; Gard, A.L.; Liu, A.; Leiby, K.L.; Schwan, J.; Kunkemoeller, B.; Calle, E.A.; Sivarapatna, A.; Lin, T.; Dimitrievska, S. Production of decellularized porcine lung scaffolds for use in tissue engineering. *Integr. Biol.* **2015**, *7*, 1598–1610. [CrossRef]
38. Johnson, P.; Trybala, A.; Starov, V.; Pinfield, V.J. Effect of synthetic surfactants on the environment and the potential for substitution by biosurfactants. *Adv. Colloid Interface Sci.* **2021**, *288*, 102340. [CrossRef]
39. van Zyl, E.M.; Coburn, J.M. Functionalization of Bacterial Cellulose with the Antimicrobial Peptide KR-12 via Chimerical Cellulose-Binding Peptides. *Int. J. Mol. Sci.* **2024**, *25*, 1462. [CrossRef]

40. van Zyl, E.M.; Kennedy, M.A.; Nason, W.; Fenlon, S.J.; Young, E.M.; Smith, L.J.; Bhatia, S.R.; Coburn, J.M. Structural properties of optically clear bacterial cellulose produced by Komagataeibacter hansenii using arabitol. *Biomater. Adv.* **2023**, *148*, 213345. [CrossRef]
41. Osborne, J. Notes on the use of data transformations. *Pract. Assess. Res. Eval.* **2002**, *8*, 6.
42. Calle, E.A.; Hill, R.C.; Leiby, K.L.; Le, A.V.; Gard, A.L.; Madri, J.A.; Hansen, K.C.; Niklason, L.E. Targeted proteomics effectively quantifies differences between native lung and detergent-decellularized lung extracellular matrices. *Acta Biomater.* **2016**, *46*, 91–100. [CrossRef] [PubMed]
43. Gervajio, G.C. *Fatty Acids and Derivatives from Coconut Oil*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2013; pp. 445–482.
44. Park, K.; Lim, J. Synthesis of phospholipid based zwitterionic surfactant from coconut oil source and characterization of their interfacial, antiseptic and antiviral properties. *J. Ind. Eng. Chem.* **2022**, *115*, 241–250. [CrossRef]
45. De, A.; Guin, M.; Jain, P. Sugar-Based Surfactants. Surfactants from Renewable Raw Materials; CRC Press: Boca Raton, FL, USA, 2021; pp. 75–96.
46. Ananthapadmanabhan, K.P.; Moore, D.J.; Subramanyan, K.; Misra, M.; Meyer, F. Cleansing without compromise: The impact of cleansers on the skin barrier and the technology of mild cleansing. *Dermatol. Ther.* **2004**, *17*, 16–25. [CrossRef]
47. Solodkin, G.; Chaudhari, U.; Subramanyan, K.; Johnson, A.W.; Yan, X.; Gottlieb, A. Benefits of mild cleansing: Synthetic surfactant based (syndet) bars for patients with atopic dermatitis. *Cutis* **2006**, *77*, 317–324. [PubMed]
48. Burnett, C.L.; Bergfeld, W.F.; Belsito, D.V.; Hill, R.A.; Klaassen, C.D.; Liebler, D.; Marks, J.G.; Shank, R.C.; Slaga, T.J.; Snyder, P.W.; et al. Final Report of the Cosmetic Ingredient Review Expert Panel on the Safety Assessment of Cocamidopropyl betaine (CAPB). *Int. J. Toxicol.* **2012**, *31* (Suppl. S4), 77S–111S. [CrossRef]
49. Clendennen, S.K.; Boaz, N.W. Betaine Amphoteric Surfactants-Synthesis, Properties, and Applications. In *Biobased Surfactants: Synthesis, Properties, and Applications*; AOCS Press: Champaign, IL, USA, 2019; pp. 447–469. [CrossRef]
50. Hunter, J.E.; Fowler, J.F., Jr. Safety to human skin of cocamidopropyl betaine: A mild surfactant for personal-care products. *J. Surfactants Deterg.* **1998**, *1*, 235–239. [CrossRef]
51. Salomon, G.; Giordano-Labadie, F. Surfactant irritations and allergies. *Eur. J. Dermatol.* **2022**, *32*, 677–681. [CrossRef]
52. Fiume, M.M.; Heldreth, B.; Bergfeld, W.F.; Belsito, D.V.; Hill, R.A.; Klaassen, C.D.; Liebler, D.; Marks, J.G.; Shank, R.C.; Slaga, T.J.; et al. Safety Assessment of Decyl Glucoside and Other Alkyl Glucosides as Used in Cosmetics. *Int. J. Toxicol.* **2013**, *32* (Suppl. S5), 22S–48S. [CrossRef]
53. Moosavi-Movahedi, A.A. Thermodynamics of protein denaturation by sodium dodecyl sulfate. *J. Iran. Chem. Soc.* **2005**, *2*, 189–196. [CrossRef]
54. Singer, M.M.; Tjeerdema, R.S. Fate and Effects of the Surfactant Sodium Dodecyl Sulfate. In *Reviews of Environmental Contamination and Toxicology*; Ware, G.W., Ed.; Springer: New York, NY, USA, 1993; pp. 95–149.
55. Yadav, V.K.; Khan, S.H.; Choudhary, N.; Tirth, V.; Kumar, P.; Ravi, R.K.; Modi, S.; Khayal, A.; Shah, M.P.; Sharma, P.; et al. Nanobioremediation: A sustainable approach towards the degradation of sodium dodecyl sulfate in the environment and simulated conditions. *J. Basic Microbiol.* **2022**, *62*, 348–360. [CrossRef]
56. Bondi, C.A.; Marks, J.L.; Wroblewski, L.B.; Raatikainen, H.S.; Lenox, S.R.; Gebhardt, K.E. Human and environmental toxicity of sodium lauryl sulfate (SLS): Evidence for safe use in household cleaning products. *Environ. Health Insights* **2015**, *9*, EHI.S31765. [CrossRef] [PubMed]
57. Abdul Rub, M.; Anamul Hoque, M.; Azum, N.; Mahbub, S. Investigation of the aggregation, clouding and thermodynamics of the mixture of sodium alginate with sodium dodecyl sulfate and triton X-100 in aqueous and aqua-organic mixed solvents media. *J. Mol. Liq.* **2022**, *346*, 117109. [CrossRef]
58. Abu-Ghunmi, L.; Badawi, M.; Fayyad, M. Fate of Triton X-100 Applications on Water and Soil Environments: A Review. *J. Surfactants Deterg.* **2014**, *17*, 833–838. [CrossRef]
59. Robson, R.J.; Dennis, E.A. The size, shape, and hydration of nonionic surfactant micelles. Triton X-100. *J. Phys. Chem.* **1977**, *81*, 1075–1078. [CrossRef]
60. Giacomelli, C.E.; Vermeer, A.W.P.; Norde, W. Micellization and Adsorption Characteristics of CHAPS. *Langmuir* **2000**, *16*, 4853–4858. [CrossRef]
61. Kroflič, A.; Šarac, B.; Bešter-Rogač, M. Thermodynamic Characterization of 3-[(3-Cholamidopropyl)-dimethylammonium]-1-propanesulfonate (CHAPS) Micellization Using Isothermal Titration Calorimetry: Temperature, Salt, and pH Dependence. *Langmuir* **2012**, *28*, 10363–10371. [CrossRef]
62. Fuller, M.E.; Andaya, C.; McClay, K. Evaluation of ATR-FTIR for analysis of bacterial cellulose impurities. *J. Microbiol. Methods* **2018**, *144*, 145–151. [CrossRef] [PubMed]
63. Smith, B. The C = O bond, part VI: Esters and the rule of three. *Spectroscopy* **2018**, *33*, 20–23.
64. Lievens, C.; Mourant, D.; He, M.; Gunawan, R.; Li, X.; Li, C.-Z. An FT-IR spectroscopic study of carbonyl functionalities in bio-oils. *Fuel* **2011**, *90*, 3417–3423. [CrossRef]
65. Adak, S.; Banerjee, R. A green approach for starch modification: Esterification by lipase and novel imidazolium surfactant. *Carbohydr. Polym.* **2016**, *150*, 359–368. [CrossRef]
66. Eilers, P.H. A perfect smoother. *Anal. Chem.* **2003**, *75*, 3631–3636. [CrossRef] [PubMed]
67. Eilers, P.H. Parametric time warping. *Anal. Chem.* **2004**, *76*, 404–411. [CrossRef] [PubMed]

68. Abol-Fotouh, D.; Hassan, M.A.; Shokry, H.; Roig, A.; Azab, M.S.; Kashyout, A.E.-H.B. Bacterial nanocellulose from agro-industrial wastes: Low-cost and enhanced production by Komagataeibacter saccharivorans MD1. *Sci. Rep.* **2020**, *10*, 3491. [CrossRef] [PubMed]
69. Wang, S.-S.; Han, Y.-H.; Ye, Y.-X.; Shi, X.-X.; Xiang, P.; Chen, D.-L.; Li, M. Physicochemical characterization of high-quality bacterial cellulose produced by Komagataeibacter sp. strain W1 and identification of the associated genes in bacterial cellulose production. *RSC Adv.* **2017**, *7*, 45145–45155. [CrossRef]
70. Zeng, M.; Laromaine, A.; Roig, A. Bacterial cellulose films: Influence of bacterial strain and drying route on film properties. *Cellulose* **2014**, *21*, 4455–4469. [CrossRef]