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ORIGINAL ARTICLE

Assessment of Culture Systems to Produce Bacterial Cellulose with a Kombucha Consortium

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Abstract
Bacterial cellulose (BC) is an emerging material for high-end applications due to its biocompatibility and physicochemical characteristics. However, the scale-up production of this material is still expensive, with the culture medium constituting one-third of the total cost. Herein, four different media (yeast nitrogen base, YNB; Murashige and Skoog, MSO; black tea; and NPK fertilizer solution) were compared while using sucrose as an additional carbon source. The yields of BC were best for YNB and fertilizer with 0.37 and 0.34 g_{BC}/g_C respectively. These two were then compared using glucose as a carbon source, with improvements in the production of 29% for the fertilizer, while only an 8% increase for YNB was seen; however, as the carbon concentration increased with a fixed N concentration, the yield was lower but the rate of production of BC increased. The obtained BC films were sanitized and showed low molecular weight and all the expected cellulose characteristic FT-IR bands while SEM showed nanofibers around 0.1 µm. Compared to traditional methods for lab-scale production, the use of the fertilizer and the consortium represent benefits compared to traditional lab-scale BC culture methods such as a competitive cost (two times lower) while posing resilience and tolerance to stress conditions given that it is produced by microbial communities and not with a single strain. Additionally, the low molecular weight of the films could be of interest for certain coating formulations.

Keywords Biopolymer · Bacterial cellulose · Low molecular weight · Growth media

Introduction

Bacterial cellulose (BC) is a versatile material, commonly synthesized as pure biofilms, with appealing properties such as a high degree of porosity, relative high permeability to liquids and gases, high water uptake, high tensile strength, high resistance, high capacity to absorb liquids, an ultrafine network, and biocompatibility [1, 2]. Thus, BC has found a wide range of applications in fashion; in food industry as a stabilizing and thickening agent [1, 3]; in biotechnological enzyme immobilization [4]; in cosmetics [5]; and in nanoelectronics for semiconductors due to good optical and photochemical properties [6, 7]. Due to its purity compared

Extended author information available on the last page of the article

to the cellulose isolated from plants, a particularly well-established niche for BC is foreseen in biomedical applications ranging from wound dressings, controlled drug delivery, biosensors/bioanalysis, stem cell therapy, skin tissue repair, surgery, and dental implants [1, 8, 9].

As BC is generated by microorganisms, it can be produced in bioreactors with controlled incubation temperature, shear force, oxygen, and pH. However, to achieve large-scale industrial production and applications, it is necessary to optimize the manufacturing processes and to study variables affecting the yields and costs. Among all the supplies required to produce BC, the culture medium represents approximately 30% of the total cost [10]. Therefore, one important and challenging aspect of the fermentation process is the identification of cost-effective culture media that can facilitate the production of high yields within short periods of time [11]. Other key factors to obtain high CB yields are the carbon and nitrogen sources [12–15]. As such, carbon sources other than the base media formulation are also added to increase the yield, with carbohydrates, including glucose (the most readily metabolized sugar), sucrose, fructose, *D*-mannitol, *D*-arabitol, and glycerol, and some alcohols such as ethanol, or glycerol and carboxymethylcellulose commonly used as additives [8, 16, 17]. On the other hand, yeast extract is the most reported source of nitrogen. Other sources include peptone, tryptone, corn liquor, and ammonium sulfate [18]. The addition of sodium or potassium phosphate is also common and helps to regulate pH changes, while magnesium chloride or magnesium sulfate is also added for the same purposes [19].

The present study aims to select a robust cost-efficient culture media-carbon source system with good BC yields using the Kombucha consortium under static culture conditions. First, two common culture media (yeast nitrogen base, YNB, and Murashige and Skoog, MSO) and two alternative media (black tea and NPK fertilizer solution) were used with sucrose as an additional carbon source. The best media, YNB and NPK fertilizer solution, were then used with a readily metabolized carbon source, glucose, to find the best carbon-nitrogen ratio (C/N). The biofilms were recovered, sanitized, and characterized via scanning electron microscopy (SEM) and Fourier transform infrared (FT-IR) spectroscopy to assess quality. The molecular weight was determined for the best BC film and a comparison for the costs of production and purification for the BC films produced from YNB and the fertilizer was done. This aimed to identify a suitable system for industrial-scale production of BC, thereby facilitating its broader utilization in emerging applications especially those where low molecular weights are required.

Materials and Methods

Microorganisms

The consortium from a Kombucha biofilm starter (Original™, GT'S Living Foods) was used as inoculum to obtain BC. It was propagated on black tea for 12 days at 28 °C and maintained at −80 °C in glycerol 20% (v/v). A sample of the consortium was activated and used to prepare the inoculum for the culture in different media.

Culture Media

Four different culture media were used:

(1) Black tea (La Pastora ®), the infusion was prepared with two bags (1.2 g per bag) in 1 L of ultrapure water.
(2) Yeast nitrogen base (YNB) without amino acids and ammonium sulfate (Sigma-Aldrich®, Milwaukee, WI, USA) with the following composition: macronutrients (g L^{-1}) KH_2PO_4 1.0, $MgSO_4$ 0.5, NaCl 0.1, $CaCl_2$ 0.1; trace elements (μg L^{-1}) H_3BO_3 500, $CuSO_4$ 40, KI 100, $FeCl_3$ 200, $MnSO_4$ 400, Na_2MoO_4 200, $ZnSO_4$ 400; and vitamins (μg L^{-1}) biotin 2, calcium pantothenate 400, folic acid 2, inositol 2000, niacin 400, *p*-aminobenzoic acid 200, pyridoxine hydrochloride 400, riboflavin 200, thiamine hydrochloride 400. The culture medium was added with $(NH_4)_2SO_4$ as nitrogen source according to the C/N ratio as required for the experiments.
(3) Modified Murashige and Skoog medium (MSO) with the following composition macronutrients (g L^{-1}): KH_2PO_4 1.3, $MgSO_4{\cdot}7H_2O$ 0.38, $CaSO_4{\cdot}2H_2O$ 0.25, $CaCl_2$ 0.055; trace elements (μg L^{-1}) $FeSO_4{\cdot}7H_2O$ 60, $MnSO_4{\cdot}7H_2O$ 48, $ZnSO_4{\cdot}7H_2O$ 52, $CuSO_4{\cdot}7H_2O$ 9.2, and $CoCl_2{\cdot}6H_2O$. The culture medium was added with $NaNO_3$ as nitrogen source according to the C/N ratio as required for the experiments.
(4) NPK fertilizer stock solution prepared by dissolving 4 g of fertilizer per liter of H_2O (20-20-20 General Purpose, Peters Professional®) macronutrients (g L^{-1}) total N 0.8 (ammonium 0.16, nitrate 0.24, urea 0.4), P 0.8, K 0.8; micronutrients (mg L^{-1}) Mg 2, Fe 2, Mn 1; and trace elements (μg L^{-1}) B 272, Cu 144, Mo 36, Zn 100, derived from $(NH_4)_3PO_4$, KNO_3, Urea, $MgSO_4$, Fe-EDTA, Mn-EDTA, H_3BO_3, Cu-EDTA, Na_2MoO_4, Zn-EDTA. The culture medium was formulated based on the total nitrogen source (fixed) according to the C/N ratio as required for the experiments.

The acidity of the media was adjusted to $pH = 5.0 \pm 0.2$ with 1 M HCl. The culture media were autoclaved at 121 °C for 15 min, and then cooled to ambient temperature prior to inoculation.

Bacterial Cellulose Production with Different Culture Media and Carbon Sources

Effect of Different Culture Media with Sucrose as a Carbon Source

In an initial experiment, the culture of BC was performed in static conditions using sucrose (20 g L^{-1}) as a very low cost and readily available carbon source. The effect of the four different culture media (black tea, YNB, MSO, and fertilizer) was evaluated with a C/N ratio adjusted to 10 for all the media except for black tea (the preferred option among kombucha brewers) as it was used without further elemental analysis. All the samples were cultured in beakers with an area/volume ratio $A/V = 0.21$ cm^{-1} (volume of culture medium = 200 mL) and were inoculated with 1 cm^2 of the Kombucha biofilm consortium. The samples were incubated in static conditions at 30 °C, for 17 days. After incubation, the biofilms were recovered from the culture media and were thoroughly washed with water followed by the removal of excess water with absorbent paper, sanitization, and characterization as described below. Yields were calculated from the final biofilm weight upon elimination of unbounded water in an oven at 50 °C for 5 days and reported as grams of BC per-gram of carbon (g_{BC}/g_C). All experiments were performed in triplicate.

Effect of Different C/N Ratios with YNB as One of the Best Culture Media with Glucose

YNB was selected to study the C/N effect on the production of BC as among all the studied media, it was one of the most homogeneous and showed one of the best yields and films in this work as described below. Three different C/N ratios (10, 20, and 30 corresponding to 20, 40, and 60 $g_{Glucose}$/L) were evaluated over time using glucose (a readily metabolized sugar) with the same A/V, temperature, and time as described above. Samples were collected and washed, and yields were calculated as described above. The maximum BC production rate was calculated with the integrated Gompertz Model, $V_{max} = 0.368\ \alpha\ k$, where α is the maximum BC produced (mgCB) and k is the CB production rate constant (h^{-1}). The parameters of the model (supporting information, table S1) were calculated using the OriginPro 8SR0 software.

BC Production with Fertilizer and Glucose as a Carbon Source at the Best C/N Ratio

BC was produced with the fertilizer and glucose as a carbon source to compare the results to the production of YNB and glucose (20 g L^{-1}, C/N 10). The culture conditions for the biofilms were the same as described in the previous experiments. To analyze the biofilms and calculate yields, destructive random samples from 30 static culture experiments were removed from the culture beakers at different times of growth. Once recovered, the excess water of the biofilms was eliminated as described above and yields were calculated and reported as grams of BC per gram of carbon (g_{BC}/g_C). Furthermore, an aliquot (1.5 mL) of the broth of each sample was recovered to quantify both glucose and ethanol during the kinetics of BC production using biochemical analysis as described below.

Media Characterization

Glucose and Ethanol Quantification

Prior to the analysis, samples were centrifuged (8000 × g for 5 min at 4 °C). The glucose and ethanol contents were determined in the supernatant in duplicate with a YSI 2900 Biochemistry Analyzer.

Organic Acid Quantification by High-Performance Liquid Chromatography (HPLC)

Organic acid analysis was performed in an HPLC (ProStar, Varian) equipped with an Aminex HPX-87-H column (300 × 7.8 mm ID; Bio-Rad) and an oven set at 50 °C under isocratic conditions with 0.005 M H_2SO_4, pH 2.0 as a mobile phase. The flow rate was 0.6 mL/min and the injection volume 20 µL. A refractive index detector (Smartline 2300, Knauer) and a UV-vis detector (ProStar 320, Varian) were used. Calibration was done with a mixed solution as follows (mg/mL): 5 formic acid, 5 acetic acid, and dilutions of 1:5, 1:10, 1:50, 1:100, and 1:1000. The samples were centrifuged at 5488 × g for 10 min, and the supernatant was filtered through a 0.22-µm nylon filter [20].

Sanitization and Bleaching of the BC Films

Prior to FT-IR and SEM characterization, the BC films were washed with water followed by sanitization and bleaching with 10% (v/v) commercial chlorine (NaClO 5.25% v/v) solution for 3 min. After sanitization, the samples were thoroughly washed with water to remove residual chlorine. The samples were pre-dried by immersing in ethanol 96% (v/v) followed by drying in an oven at 50 °C for 5 days.

Characterization of Bacterial Cellulose

Fourier Transform Infrared (FT-IR) Spectroscopy Analysis

The BC films produced in the static medium were sanitized and characterized to verify their cellulosic nature by FT-IR and conformation through SEM as described below.

The FT-IR (ATR) spectra of dry sanitized BC samples were obtained on a Bruker Tensor 27 FT-IR spectrometer (Bruker Optik, Ettlingen, Germany) in transmission mode. The OPUS software (Version 6.0) was used for spectra acquisition and processing. The spectral range of analysis was from 4000 to 500 cm^{-1}.

Scanning Electron Microscopy (SEM)

The biofilms were mounted onto SEM stubs using conductive carbon adhesive tabs and colloidal graphite and were sputter coated with gold in a Denton Vacuum, LLC sputter coater. SEM images were acquired for non-sanitized and sanitized BC biofilms through secondary electron imaging mode in a JSM 5900 LV (Jeol) Microscope.

Gel Permeation Chromatography (GPC)

Molecular weights were determined by GPC/SEC on HPLC (ProStar, Varian) equipped with a column of 10 µm PLgel MIXED-B (300×7.5 mm ID; Agilent). The sample was filtered with a 0.45-µm nylon filter. The injection sample volume was 20 µL. The mobile phase was N,N-dimethylacetamide with 0.9% (w/v) LiCl, filtered with 0.45-µm nylon filter. The flow rate was 1.0 mL min^{-1} at 40 °C and detection was performed using a refractive index detector (Smartline 2300, Knauer). Two independent calibration curves with polystyrene (PL2010-0100, Agilent Technologies) molecular weight range of 3,053,000 to 580 Da and pullulan polysaccharide kit standards (PL2090-0100, Agilent Technologies) with molecular weight range of 107,000 to 180 Da were used to calibrate the system. Chromatograms were analyzed with the GPC Software extension ClarityChrom 3.1.

Statistical Analysis

A one-factor analysis of variance (ANOVA) ($p < 0.05$) and a Tukey test were used to determine significant differences among the yields in the four-culture media and to identify significant differences at a confidence level of 95%. The same tests were used to compare the yields of BC using glucose as a carbon source in the YNB medium at three concentrations

(20, 40, and 60 g/L). To determine the differences in yields when using saccharose and glucose in YNB and fertilizer, an ANOVA ($p < 0.05$) was used.

Results and Discussion

Comparison of the Different Culture Media with Sucrose as a Carbon Source

A path to enhance the production of BC while lowering the cost is by selecting the best culture medium to increase the yield. Thus, four culture media were compared keeping the carbon source constant with the commercially available sucrose. The carbon-nitrogen ratio (C/N) was kept constant at 10 and the area/volume ratio of the contained was 0.21 cm^{-1} for all the experiments. The yields of BC are presented in Table 1, and Fig. 1 shows the BC membranes prior to any post-treatment.

Overall, the best culture media to produce BC were YNB and the fertilizer showing comparable yields of around 0.37 and 0.34 g_{BC}/g_C, respectively. In contrast, the yields from black tea and MSO were approximately three times lower. The Tukey test shows no significant difference in the yields when using the YNB and the fertilizer. However, there is a difference in the yields from these two media compared to the black tea and MSO.

The yields obtained herein using the Kombucha consortium with sucrose as a carbon source and YNB or fertilizer as culture media were comparable to those reported for *A. xylinum* using mannitol with a yeast extract medium, peptone, Na_2HPO_4, and citric acid [17]. Yields were also comparable to the ones from *G. xylinus* with glucose and 1% ethanol with Hestrin-Schramm (HS) medium [21]. Compared to this work, Pa'e et al. [22] obtained a similar productivity with lower yields (0.05 g_{BC}/g_C) when using 50 g/L of sucrose with Yamanaka medium and *A. xylinum*. Thus, when using commercially available sources to produce BC, the yields obtained with the consortium are competitive with those of pure strains, with the additional advantage of decreasing purification needs and special conditions for a single strain.

The response to different media variables is highly strain dependent, with the most studied strains for large-scale BC production being *A. xylinum* and *G. xylinus* [8, 23]. However, the production of BC with a single strain requires high-quality standards to maintain

Table 1 Yields and productivities of BC with different carbon sources and using various culture media

Carbon source	Concentration (g/L)	Culture medium	Yield (g_{BC}/g_C)	Productivity (g_{BC}/L day)
Sucrose	20	YNB	0.37 ± 0.06	0.21
		Fertilizer	0.34 ± 0.06	0.16
		Black tea	0.11 ± 0.03	0.08
		MSO	0.11*	0.02
Glucose	20	Fertilizer	0.44 ± 0.02	0.19
	20	YNB	0.40 ± 0.01	0.18
	40		0.33 ± 0.03	0.29
	60		0.23 ± 0.03	0.30

*A BC film was obtained only in one of the replicates

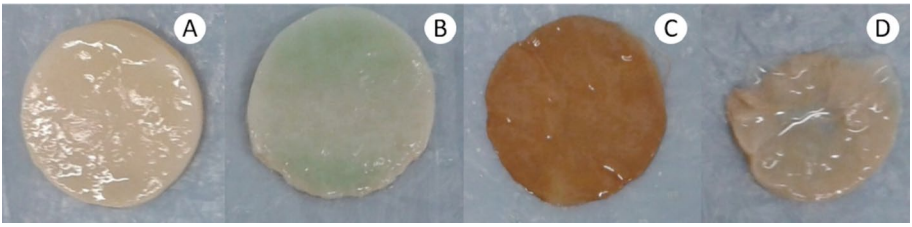

Fig. 1 Bacterial cellulose films produced with sucrose and different culture media, **A** YNB, **B** fertilizer, **C** black tea, and **D** Murashige and Skoog, with a C/N ratio adjusted to 10 for all the media except for black tea

the strain and to avoid cross-contamination. This increases cost and time and lowers the robustness of the process for large-scale production. In this way, the production of BC with a consortium is more robust. Furthermore, the metabolic limitations of single organisms are avoided with the consortium, and resilience to perturbations is gained as it has been observed that in communities of diverse microorganisms, there could be a varying tolerance to stress conditions. Good BC outputs have been found even when not using single strains as is the case of this work [24, 25]. In Kombucha, a mutualistic relationship between acetic acid-producing bacteria and yeasts is established [26, 27]. The bacteria and the yeast grow in two mutually non-exclusive parts of the fermented drink, the biofilm that forms and floats at the air-culture medium interface and the liquid (broth). Biofilms produced by microorganisms work as an adaptive mechanism (floating device) that allows aerobic bacteria to thrive at the air-liquid interface and obtain the necessary oxygen to grow. These biofilms also constitute the physical barrier that protects the microorganisms from UV radiation and allows the retention of humidity [28]. Compared to the biofilms, the microbial diversity is higher in the liquid [29], with some of the microorganisms identified in Kombucha being *Gluconacetobacter xylinus* (reclassified as *Komagataeibacter xylinus*), *Zygosaccharomyces* sp., *Saccharomyces* sp., *Pichia* sp., *Saccharomyces pastorianus* Hansen, *Schizosaccharomyces pombe* Lindner, *Acidomonas methanolica*, and *Lactobacillus*, among others [30].

To have a better understanding of the effect of the media on the properties such as the color of the produced films, a RGB color identification was done, and the results are shown in Table 2. Except for the films from the fertilizer, all films had a light brown pigmentation in different shades and saturation. The darkest films were those obtained from black tea due to their natural tannin constituents (thearubigins and theaflavins). Of all the culture media used, the films with the best appearance (more homogeneous surface and less pigmentation) were those from the YNB medium. The films from the fertilizer had blue-green zones (Fig. 1B, Table S2) derived from the color of the fertilizer and were probably related to the higher content of Cu (144 µg/L) compared to YNB (40 µg/L) in its formulation.

BC Production with the Best Culture Media and Glucose as a Carbon Source

From the initial experiments, the best culture media were YNB and the fertilizer; therefore, both media were selected to continue the study with an alternative carbon source, namely glucose, at a C/N 10 corresponding to 20 g/L of glucose. At this ratio, compared to the initial experiment with sucrose, the fertilizer yield was 29% higher, and the YNB was improved by 8% when glucose was used (Table 1).

Table 2 Associated cost of the production of the bacterial cellulose films

Substance	Cost USD	Qty	Cost per film (USD)
YNB	2000.19/kg	Medium 200 mL (3.4×10^{-4} kg)	0.68
Fertilizer	14.32/kg	Medium 200 mL (8×10^{-4} kg)	0.011
D-Glucose	60.70/kg	20 g /L Medium 200 mL (4×10^{-3} kg)	0.24
Innoculum	20.38 (56.7 cm^2)	1 cm^2	0.35
Incubation	17 days (30 °C)	1.32 USD (30 films)	0.044
Drying	5 days (50 °C) 0.063 USD/h	7.6 USD (50 films)	0.15

Figure 2 shows the kinetics of BC production comparing the fertilizer and YNB media and glucose as the carbon source. The maximum production rate of BC with glucose and the fertilizer (V_{max} 2.0 mg_{BC} h^{-1}) was comparable to that for the culture in the YNB medium (V_{max} 2.8 mg_{BC} h^{-1}). The yields correspond to 0.44 g_{BC}/g_C and 0.4 g_{BC}/g_C for the fertilizer and YNB medium, respectively. Statistically comparing the carbon sources (sucrose and glucose) and the media at a concentration of 20 g/L (C/N 10), the ANOVA test shows that there is a significant difference between the carbon sources but not between the media (YNB and fertilizer).

On the other hand, the biochemical analysis results indicated that as the metabolism proceeded, glucose was consumed completely after 300 h (supporting information, Figure S1). Up to this point, the production of ethanol increased (Fig. 3). Ethanol was subsequently consumed, when the conventional carbon source (glucose) was consumed; therefore, the ethanol that accumulated during the fermentation acted as an alternative carbon source and was consumed after 300 h.

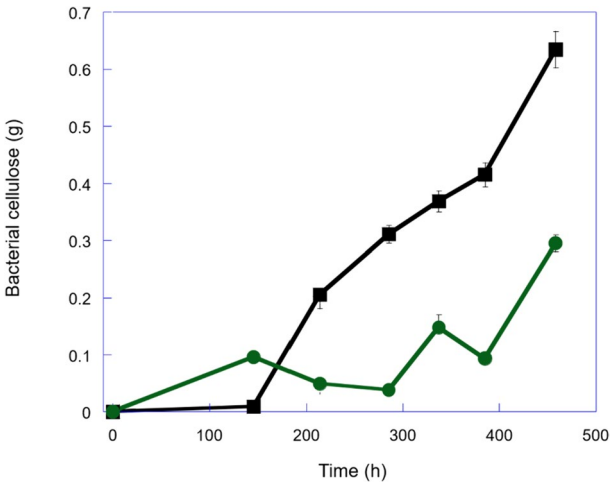

Fig. 2 BC production in static culture with fertilizer (squares) and YNB (circles) using glucose as a carbon source

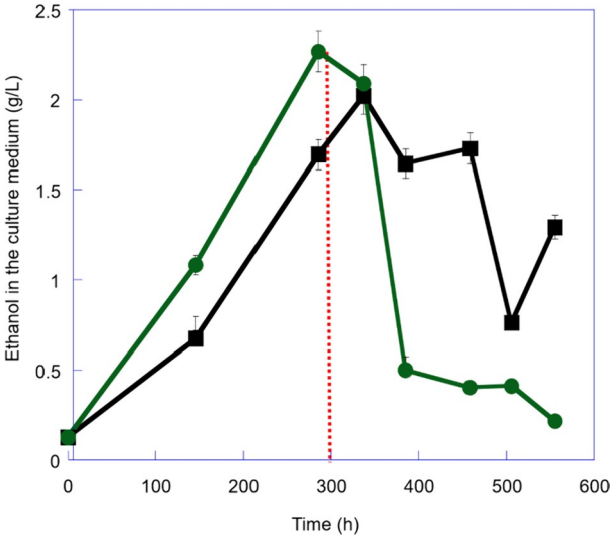

Fig. 3 Ethanol production and consumption on static culture in fertilizer (squares) and YNB medium (circles) with glucose as a carbon source

Effect of Different C/N Ratios with YNB as One of the Best Culture Media

To increase the YNB yield with glucose, different concentrations of it were compared; this effect can be visually seen in Fig. 4. As the concentration of carbon (glucose) increased from 20 to 40 g/L and 60 g/L (C/N 10, 20, and 30, respectively), the yield decreased from 0.4 to 0.23 g_{BC}/g_C, while the productivity rose from 0.18 to 0.29 g_{BC}/L·day and up to 0.3 g_{BC}/L·day, i.e., almost double the one for the lowest glucose concentration. Once statistically compared, there was a significant difference between the bacterial cellulose yields when using glucose in the YNB medium at the three evaluated concentrations.

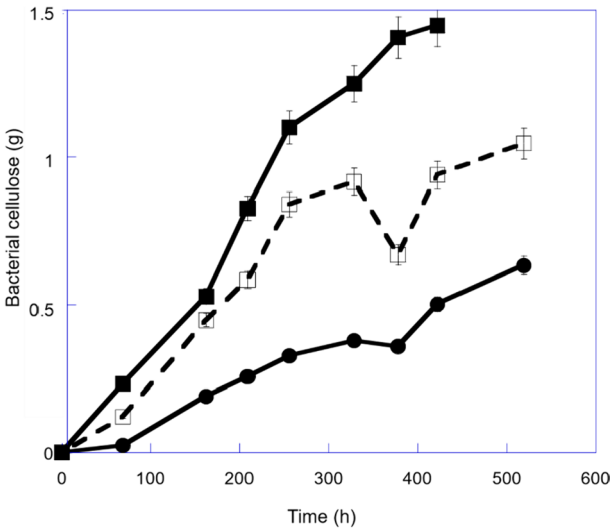

Fig. 4 Effects of C/N on the production of BC in YNB with glucose as a carbon source; C/N 30 (closed squares), C/N 20 (open squares), and C/N 10 (closed circles)

The calculated V_{max} with the Gompertz model shows an increase from 1.4 to 4.2 mg_{BC} h^{-1} and up to 5.1 mg_{BC} h^{-1} for the three C/N ratios (Table S1).

As the carbon concentration increased with a fixed N concentration, the yield was lower but the rate of production of BC increased. Therefore, according to these results, the most optimal C/N ratios (i.e., the one with the best yield and the best production rate) were 10 and 20. The C/N 30 shows a comparable production rate, but lower yield. In general, C/N 10 and 20 use less carbon (lower energy use) with better results that are comparable to the ones using YNB and sucrose.

Comparison Between the Formed BC Films

After microbial production, BC is not pure as it contains some of the microorganisms of the consortium, which the SEM images of the membranes confirmed (Fig. 5). The presence of these microorganisms can be detrimental for some applications, especially for biomedicine or the food industry. When the media was tested for organic acids, concentrations of up to 0.8 g/L of acetic acid were measured, while the presence of other organic acids was not observed. The acetic acid produced could be a valuable by-product that can be recovered from the cultures in the BC bioprocess.

Most of the protocols to purify BC are essentially based on washing with alkalis or acids like NaOH 2% (w/v) and KOH (2–5% w/v) followed by neutralization with water. Some other methods require various chemicals including NaOH/KOH/Na_2CO_3 at 100 °C for 15–20 min to lyse the microbial cells, followed by filtration and rinsing with water upon neutralization [31]. Other authors report two purification steps: NaOH 2.5 wt% followed by NaClO 2.5 wt% (bleaching) [32]. Tsalagkas et al. [33] reported the use of NaOH 2.5 wt% (6×10^{-3} M) as a one-step purification, studied the two-step purification reported by Gea et al. [32], and finally used 0.1 M NaOH at 70 °C for 2 h under stirring. The best of the treatments was the hot 0.1 M NaOH solution. This work explored the removal of microorganisms using a commercial chlorine 10% v/v (NaClO 5.25% v/v solution) as an economic sanitization method. The SEM images prior and after sanitization show the successful elimination of microorganisms. Chlorine (NaClO) is accepted by the US Environmental Protection Agency and the US Department of Agriculture for use as disinfectant and in safe food production and is therefore a good candidate for sanitization of the BC biofilms. However, further sterilization could be necessary for the films to be used in biomedical industry applications.

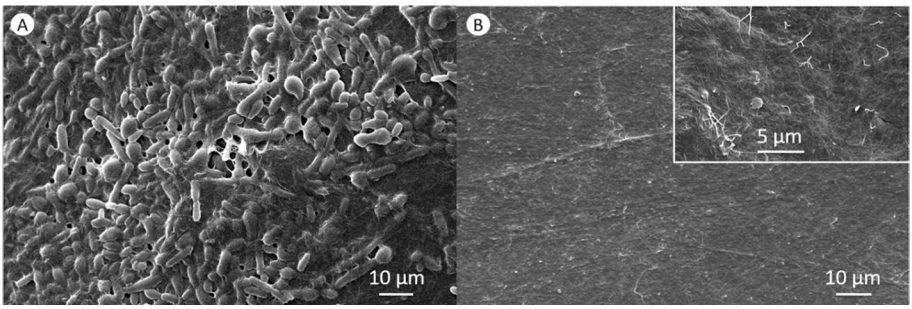

Fig. 5 SEM images of BC **A** prior and**B** after sanitization treatment with chlorine

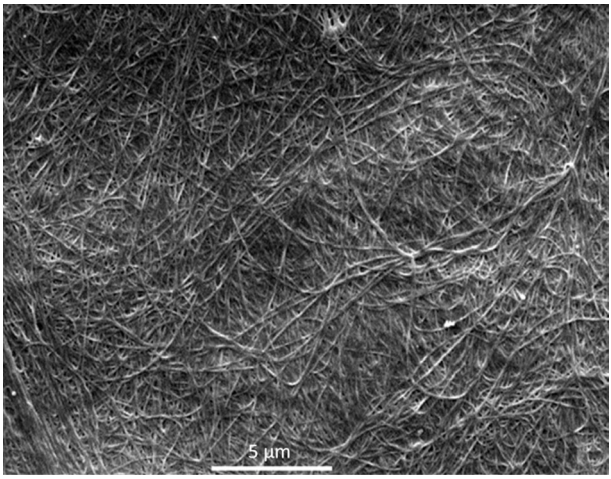

Fig. 6 SEM image of BC fibers

It is worth mentioning that chlorine allowed for the simultaneous bleaching of the biofilm (supporting information, Figure S2). It is common to bleach cellulose using chlorinated compounds [34]. Chlorine is the most used high-oxidizing agent that removes pigments [35]. Chlorine in combination with water formed hydrochloric acid and O_2 that degraded the pigments of the produced BC films.

Fibrils, also known as nanofibers (2–10 nm), have gained attention due to properties such as high strength, stiffness, low weight, and biodegradability [36]. As can be observed from Fig. 6, the obtained bacterial cellulose was at this nanofibrillar range (100 nm).

Figure 7 shows the cross-section SEM images of BC obtained from tea, YNB, and the fertilizer. The film obtained from the MSO medium was not suitable for the cross-section analysis. As can be observed from the cross-section, the thickness of the films from YNB and the fertilizer was comparable, whereas the BC obtained from tea was three times thinner. It is clear from the results that the culture media determine the film thickness. The control of this variable can then be used to obtain films designed for different applications. Compared to other films, the BC from the YNB and fertilizer were 50% thinner [37] but thicker than BC obtained with *G. xylinus* (PTCC 1734) (10 to 30 µm), and two strains isolated from vinegar with glycerol as a carbon source in HS medium [38] and thicker to the typical BC (10 and 50 nm) [39].

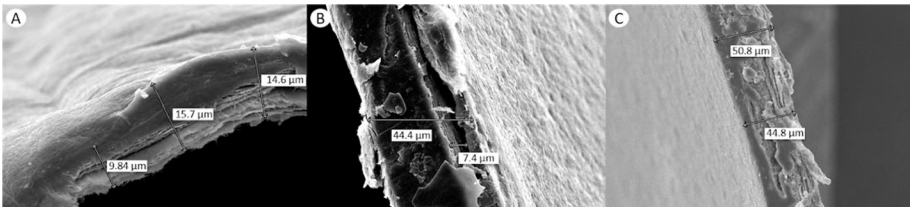

Fig. 7 Cross-section SEM images of BC produced in different culture media: **A** tea, **B** YNB, and **C** fertilizer

The BC from tea is formed by various parallel layers, in contrast to the films from YNB and the fertilizer, which are more compact. Similar multilayered films are common and have been observed previously [40]. The layers are the result of the further growth of BC at the film/air interface.

Fourier transform infrared spectra were obtained by attenuated total reflection (FT-IR-ATR) to confirm that the product was cellulose. The observed absorption bands (ν/cm^{-1}) were as follows: 3350 and shouldering from 3400 to 3500 (broad, O–H stretching), 2800–2900 (narrow, C–H stretching), 1620 (narrow, O–H absorbed H_2O bending), 1160 (asymmetrical, C–O–C stretching), 1300–1060 (C–O stretching), 1300 (planar, C–H bending), 1400 (CH_2 bending). The biofilms obtained from the best culture media showed the characteristic bands from 3800 to 2900 cm^{-1}, 2900 to 2800 cm^{-1}, 1160 cm^{-1}, and 1060 to 1035 cm^{-1} from O–H, C–H, C–O–C, and C–O groups respectively (Fig. 8) that correspond to cellulose. The fingerprint region also shows the characteristic C–H bending at around 1300 cm^{-1} and the CH_2 bending at 1400 cm^{-1} for cellulose. The band at around 1620 cm^{-1} was assigned to –OH from absorbed water [41, 42].

The molecular weights were determined for the bacterial cellulose film produced with fertilizer and glucose, which had the best yield and costs. The chromatogram showed a peak with a tail, and the retention time was higher than those for the polystyrene standards. Therefore, the pullulan standard curve was used as a reference. Upon correction of the BC molecular weights relative to pullulan standards [43], the estimated values for the first peak and the tail were 5779.8 Da and 2578.45 Da, respectively. No other peaks were observed, indicating no degradation of the BC in the sample solvent during the GPC/SEC analysis. Although higher molecular weights are obtained from cultures of pure strains of microorganisms, low molecular weights in the range of 9000 to 10,000 Da have also been reported for cultures at low pH [44]. The consortium could have influenced the low molecular weights in this work. However, low molecular weight cellulose could be of interest for coating formulations [45].

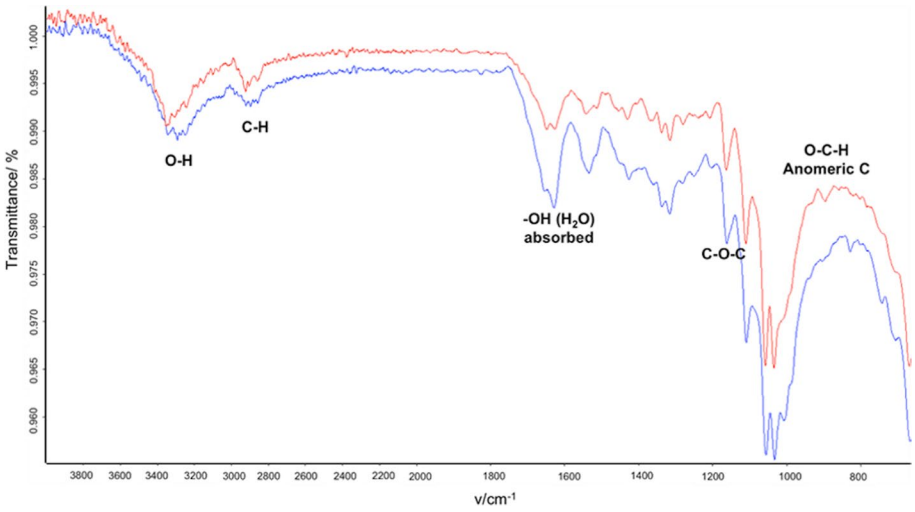

Fig. 8 FT-IR (ATR) spectra for BC obtained with fertilizer (above) and YNB (below)

Advantages of the Fertilizer and Glucose for the Production of BC

The chemical nutrients required by microorganisms to grow are Fe, K, Mg, Mn, S, N, Ca, Co, Cu, P, and Zn. Most of the available culture media contain these elements and even some vitamins to support the life of microorganisms. Except for vitamins, the fertilizer had a source for all the nutrients and following trace elements as in YNB: N, P, K, Mg, B, Cu, Fe, Mn, Mo, Zn, S, and Na. However, no sources for I and Cl_2 and Ca (found as KI, $FeCl_3$ and NaCl, $CaCl_2$, respectively) were present in the fertilizer compared to YNB. This indicates that for single strains as for the Kombucha consortium, N, P, K, Mg, B, Cu, Fe, Mn, Mo, Zn, S, and Na are critical elements for the growth of BC. Like all modern fertilizers, the one used in this study contains N, P, and K as these are the required nutrients for optimal plant growth. As mentioned above, the same nutrients are found in YNB, and considering the obtained yields, it can be assumed that all these nutrients are important for the production of BC with single strains and for the consortium. According to the yields (0.44 g_{BC}/g_C) obtained with one of the best media (fertilizer) and with the best carbon source (glucose), it was observed that the vitamins in the second best medium (YNB) were not relevant to produce BC with the consortium. All these findings suggest that the culture medium may be specifically designed to optimize large-scale production with consortia or pure strains. Nainggolan et al. [46] prepared their own culture medium by following the method from Gea et al. [19] using glucose, ammonium sulfate, potassium hydrogen orthophosphate, yeast extract, and magnesium sulfate in a liter of water and with a slightly different pH adjustment (pH 4.0 compared to pH 5.0 of the Gea et al. [19] method). However, and in contrast to this work, both groups also added vitamins B1, B2, and B5 that were not required in our study.

As discussed above, BC yields with the consortium and either YNB or the fertilizer were comparable to those reported in literature for single strains. Various sugars including monosaccharides, disaccharides and polysaccharides, alcohols, and organic acids have been used to obtain BC. Glucose seems to be the preferred carbohydrate. However, D-arabitol and D-mannitol have been described as better carbon sources giving yields of 6.2- and 3.8-folds higher respectively compared to glucose [8, 17]. As of June 2023, the prices (USD/kg) for the D-arabitol (Sigma-Aldrich, ACS reagent) ($16,181.21), D-mannitol (Sigma-Aldrich ACS reagent for microbiology) ($89.73), and D-glucose (Sigma-Aldrich ACS reagent) ($60.70) are up to 266-fold higher for D-arabitol and 3.82 for D-mannitol compared to D-glucose reagent grade. The cost for YNB ($2000.19 USD/kg) is 139.6 times higher than the cost of the fertilizer ($14.32 USD/kg). Table 2 shows a comparison between the different supplies and the cost per film, with also approximately two times decrease from the YNB (1.46 USD/film) compared to the films made with fertilizer (0.79 USD/film). The methodology described in this work yielded BC with the cheapest alternatives for medium and carbon sources. It is also worth mentioning that as the production method was performed in static conditions, there were no energy costs related to agitation.

The purification process proposed here, even though low cost (Table 3), summed to 3.86 USD per film with most of the cost coming from the water used for the washes. These washings are needed as some of the most promising applications for BC are from the biomedical field. Thus, the material needs to be free from compounds in the media that may cause side or toxic effects and that can compromise the biocompatibility.

The concentration of components in the YNB medium is described as trace elements in the range of ppb; therefore, it is presumed that the BC films pose no health risk after

Table 3 Associated cost of the cleaning and purification of bacterial cellulose films

Substance	Volume (mL)	Cost (USD/film)
10% v/v NaClO (5.25% v/v)	7	0.008
Distilled water	2000	3.49
Ethanol	50	0.21
Drying	5 days (50 °C) 0.063 USD/h	7.6 USD (50 films) 0.15 USD/film

purification. Nonetheless, the fertilizer also has elements like Fe, Mg, and Mn as micronutrients in the order of mg/L. Mo and B and Zn and Cu are also present in both culture media as trace elements. It has been reported that Mo is a potent inducer of osteogenic differentiation in human bone marrow-derived mesenchymal stromal cells. On the other hand, boron is helpful in vascularization of bone in applications of tissue engineering [47]. Therefore, both elements combine the advantages of being pro-osteogenic and pro-angiogenic having a synergistic effect in the context of regenerative medicine.

The films from the fertilizer had additional green and blue zones (Fig. 1B; Table 2) derived from the natural color of the fertilizer and probably related to a higher content of Cu (144 µg/L compared to YNB that has a content of 40 µg/L) in its formulation. The presence of certain elements like Cu in the films can be detrimental for applications especially in the biomedical field. It can be toxic and mutagenic or cause inflammatory or allergic responses. In vitro studies of the cytotoxic effects of Cu show that osteoblast like cell death is directly related to the distance of the cells to the element and the exposure time [48]. These authors found that the number of cells decreased from 40 to 12% in 8 h of culture showing a further decrease to 2% after 24 h of culture. At this final time, the morphology of the cells had also changed. Another study examined the cytotoxic effects of copper (Cu) ions on L929 mouse fibroblasts, revealing a reduction in cell viability with increasing Cu ion concentration. At 24 h, concentrations of 40 µg/ml and 100 µg/ml resulted in 30% and 100% reductions, respectively. Furthermore, a concentration of about 46 µg/ml (~29 µM) was identified as the LD50 dose for L929 mouse fibroblasts when exposed for 24 h based on our MTT cell viability assay [49]. However, the fertilizer has a concentration of only 144 µg/L. Therefore, purification of the BC films could lower even more the amount of Cu, also diminishing the health risks.

Compared to the fertilizer, YNB seems a better culture medium for these applications as it contains 3.6 less Cu. However, to use the BC films, further studies to assess the individual and synergistic effects of all the elements present in the media should be required according to the applications.

Especially for biomedical applications, it is worth emphasizing the importance of exploring the release of contaminants through experimental designs within therapeutic and cytotoxic limits. Additionally, the possibility to formulate a culture medium like that of fertilizer with safer and innocuous elements for biomedical applications is a good alternative to further explore fertilizer as a cheaper medium than YNB.

Conclusions

A novel and robust production method for BC using the Kombucha consortium in two culture media, namely YNB and a common commercial NPK plant fertilizer with glucose as a carbon source, was the best option in this study. Using the fertilizer, which contains all

the essential elements found in YNB, allowed for a cheaper production cost with similar yield. The low molecular weight of the BC obtained from the fertilizer could be of interest for some coating formulations. Furthermore, the use of commercial chlorine to sanitize BC presented a low cost and easy way to remove the microorganisms and to bleach the polymeric membranes for further applications. Thus, using the consortium with easy access and low-cost supplies allowed to produce BC in good yields for added-value applications.

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Data Availability Data will be available on reasonable request.

Declarations

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