Optimization of moist and oven-dried bacterial cellulose production for functional properties

Ioana Maria Bodea

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca

Florin Ioan Beteg

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca

Carmen Rodica Pop

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca

Adriana Paula David

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca

Mircea Cristian Dutescu

Technical University of Cluj-Napoca

Cristian Vilău

Technical University of Cluj-Napoca

Andreea Stănilă

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca

Ancuța Mihaela Rotar

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca

Giorgiana Mihaela Catunescu (✉ giorgiana.catunescu@usamvcluj.ro)

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca <https://orcid.org/0000-0002-8006-4973>

Original Research

Keywords: drug-release, SEM, swelling ratio, thickness, TEM, Response Surface Methodology

Posted Date: February 4th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-203952/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Version of Record: A version of this preprint was published on June 24th, 2021. See the published version at <https://doi.org/10.3390/polym13132088>.

Abstract

Bacterial cellulose (BC) is a natural polymer with properties suitable for tissue engineering and possible applications in scaffold production. However, current procedures have limitations in obtaining BC pellicles with the desired structural, physical, and mechanical properties. Thus, this study analyzed the optimal culture conditions of BC membranes and 2 types of processing: draining and oven-drying. The aim was to obtain BC membranes with properties suitable for a wound dressing material. Two studies were carried out. In the preliminary study the medium (100 mL) was inoculated with varying volumes (1; 2; 3; 4; and 5 mL) and incubated statically for different periods (3; 6; 9; 12; and 18 days), using a full factorial experimental design. Thickness, uniformity, weight, and yield were evaluated. In the optimization study, a Box–Behnken design was used. Two independent variables were used: inoculum volume (X1: 1; 3; and 5 mL) and fermentation period (X2: 6; 12; and 18 d) to determine the target response variables: thickness, swelling ratio, drug release, fiber diameter, Tensile strength, and Young's Modulus for both dry and moist BC membranes. The mathematical modelling of the effect of the 2 independent variables was accomplished by response surface methodology (RSM). The obtained models were validated with new experimental values, and confirmed for all tested properties, except Young Modulus of oven-dried BC. Thus, the optimal properties in terms of a scaffold material of the moist BC were obtained with an inoculum volume of 5% (v/v) and 16 d of fermentation. While, for the oven-dried membranes a 4% (v/v) and 14 d of fermentation.

Introduction

Bacterial cellulose (BC) has an array of physical, mechanical and biological properties, such as swelling behavior (Lin et al. 2013; Zeinali Kalkhoran et al. 2018), high tensile strength (Li et al. 2015b; Lin et al. 2013; Wu et al. 2018), high water-holding capability (Wu et al. 2018; Zeinali Kalkhoran et al. 2018), high porosity (Ullah et al. 2017), ultra-fine fiber network (Lin et al. 2013), biodegradability, non-toxic nature and biocompatibility makes it a promising material in tissue engineering (Badshah et al. 2017; Bodea et al. 2019; Fu et al. 2013; Rambo et al. 2008). It is produced by several bacteria as a primary metabolic product in the form of swollen membranes (Badshah et al. 2017). However, the most effective is *Komagataeibacter xylinus*, former known as *Gluconacetobacter xylinum*. It is currently used to produce commercially available BC because of its high productivity (Blanco Parte et al. 2020; Wang et al. 2019).

BC has been studied rather extensively, mainly in terms of biosynthesis and applications (Bodea et al. 2019; Wang et al. 2019). Different methods and systems to produce BC have been analyzed and many of studies dealt with the production of BC in the last decades (Ahmed et al. 2019; Bae and Shoda 2005; Bilgi et al. 2016; Du et al. 2018; Tan et al. 2012). Their main aim was to increase BC productivity by obtaining different morphologies, structures, properties, and applications. They usually tried to increasing the BC yield by employing experimental designs and statistical methods to optimize the type and proportion of culture media (Bae and Shoda 2005; Bilgi et al. 2016; Santoso et al. 2020). Response surface methodology (RSM) is one of the current approaches of the BC optimization studies and it is usually applied on a Box-Behnken Design (BBD) (Ahmed et al. 2019; Bae and Shoda 2005; Du et al. 2018; Tan et al. 2012). In terms of culture, the static method was most frequently used to obtain BC at large scale because it is a relatively simple technique (Bilgi et al. 2016; Wang et al. 2019). However, there seems to be a lack of studies that evaluated the simple inoculum volume – fermentation duration relationship in terms of their effect upon the biomedical properties of BC. Thus, it is not yet clear how these basic process parameters influence the uptake ability of BC.

Pristine BC has a unique macromolecular structure consisting of thin subfibrils, which form microfibrils because of strong inter- and intramolecular hydrogen bonding (Badshah et al. 2017). The usual purification methods of the pristine BC pellicles is the treatment with 0.1% NaOH solution for 1 h at 80°C, while the main processing of the BC is lyophilization (Kirdponpattara et al. 2015; Santoso et al. 2020; Ullah et al. 2017). However, this processing affects the properties of the native membrane such as: visco-elasticity or uptake capabilities (Alonso et al. 2018; Fu et al. 2013; Zhang et al. 2011) important characteristics for biomedical applications. Another simple and cheap processing method is oven-drying. It seems to make the membrane compact, and brittle (Fu et al. 2013; Zhang et al. 2011) because the BC structure becomes more rigid and less porous (Alonso et al. 2018). However, the effect of this processing on the biomedical properties of BC is not fully described as oven-drying of BC is reported sparingly in the scientific literature.

Thus, the aim of the present study is to determine the optimal combination of inoculum volume and fermentation duration for *G. xylinus* ATCC 700178 to produce BC membranes with desirable morphological, mechanical, and uptake properties for biomedical purposes, in particular for wound dressing scaffold. RSM was chosen to model, analyze, and optimize the properties of BC (the dependent variables) because they are simultaneously influenced by both inoculum volume and fermentation duration (the independent variables). An BBD was chosen as it is currently of great interest because it generates a smaller number of experiments with reliable results (Ahmed et al. 2019). Additionally, the effect of oven-drying was evaluated against the pristine water-drained BC using the same methodology and properties of biomedical interest. To the best of our knowledge this is the first study which intends to optimize the fermentation (inoculum volume and duration) and oven-drying of pristine BC in terms of biomedical properties by employing mathematical modelling.

Materials And Methods

Microbial strain and chemicals

Microbial strain: *Gluconacetobacter xylinus* (*Komagataeibacter xylinus*) (ATCC® 700178™); chemicals: glucose, yeast extract, CaCO₃, Agar, NaOH, NaOCl; 1.6% glutaraldehyde; sodium cacodylate trihydrate (C₂H₁₂AsNaO₅) buffer, osmium tetroxide 1%, uranyl acetate 2%.

Fermentation method

The utilized liquid and solid media both contained 50 g/L glucose, 5 g/L yeast extract and 12.5 g/L CaCO₃ (Yu et al. 2016), but the liquid had distilled water as solvent, while the solid medium had an agar base (15 g/L agar).

The lyophilized *G. xylinus* was reactivated by pouring 1.0 mL of liquid medium to rehydrate the pellet, then the suspension was transferred into a 5 mL tube, vortexed and incubated at 26°C for 72 h. The bacteria were cultured 3-4 times until a homogeneous cellulose film was observed on the surface of the medium (Cai and Kim 2009). The suspension was then cultured on sterile solid medium and incubated (DigitHeat 2001245) at 26°C for 7 days. The culture was preserved on solid medium at 4°C and recultivated as described, every 2 to 3 weeks.

The inoculum solution was prepared by aseptically removing 7 to 9 bacterial colonies of the 7-day old *G. xylinus* culture with a cell spreader and transfer them in a 9 mL sterile saline solution tube. Then the resulting solution was mixed thoroughly for 5 min using a MaxQ 2000 Vortex Mixer. The resulted bacterial cell suspension was adjusted to 1.5×10^7 cells/mL (Dahman et al. 2010) using a spectrophotometer (Shimadzu UV-1900) at 600 nm absorbance and used as inoculum solution.

The fermentation was carried out in sterilized 120 mL square glass bottles containing 100 mL specific liquid medium. For the preliminary study the medium was inoculated using varying inoculum volume (1; 2; 3; 4 and 5 mL corresponding to 1%; 2%; 3%; 4% and 5% (v/v)) and incubated statically at 26 °C for different periods (3; 6; 9; 12; and 18 days). The maximum of 5 mL was chosen because higher values decreased the cellulose production (Rangaswamy et al. 2015). A maximum chosen because although the reported fermentation period varied from 4 days (Bilgi et al. 2016) to 30 days (Zeng et al. 2011), the usual maxim was between 14 to 21 days (Bilgi et al. 2016; Du et al. 2018; Zeng et al. 2011), supporting our choice. For the optimization study a Box-Behnken Design (BBD) experimental design was used with 3 levels for both the inoculum volume (1; 3; 5 mL) and fermentation period (6; 12; 18 d) (Table 2). All samples were performed in triplicate.

Purification of bacterial cellulose (BC)

BC membranes were collected from the surface of the liquid medium and washed repeatedly (cca. 3 times) with distilled water. Then, BC was treated with 500 mL of 0.1% NaOH solution for 1 h at 80°C on a magnetic stirrer to remove all bacteria cells. The BC pellicles (6.5/6.5 mm) were washed again with distilled water and left for 24 h in a 3% NaOCl solution (Gea et al. 2011). Afterwards, they were washed with distilled water until reaching a pH of 7. The membranes thus obtained were stored either in distilled water at 4°C until further analysis (thickness, uniformity, weight, water uptake ability, and drug release) or individually vacuum packed in transparent polyethylene foil (for mechanical test, TEM, SEM and FTIR).

Processing of BC membranes

Throughout the experiment 2 types of membrane processing were analyzed: half of the batch was oven-dried (DigitHeat 2001245) at 40°C until constant weight (cca. 30-60 min), resulting dry BC pellicles (BCd) (Rambo et al. 2008), while the other half was pressed with filter paper until almost all the water in their structure was removed, resulting drained, but moist BC pellicles (BCm).

PRELIMINARY STUDY

Transmission electron microscopy (TEM)

Two types of samples (32.5 x 32.5 mm) were used for TEM analysis: pristine untreated and unpurified BC pellicles, and purified BC membranes. TEM was used for the analysis of the cellulose-forming bacteria and evaluation of the purification process. The cellulose films were treated with 1.6% glutaraldehyde (1 h fixation) and buffer (0.1 M, pH 7.4). Each sample was washed 3-5 times every 5-10 min and left in the buffer for 2 days. Then, the samples were fixed for 1h in osmium tetroxide 1%, incubated in the dark for 2h in uranyl acetate 2%, dehydrated in different concentrations of acetone and incorporated into acetone/resin in a RT agitator, then infiltrated in resin Spurr/epon 100% and polymerized. Finally, the samples were cut ultrafine and analyzed with a transmission electron microscope (ZEISS LIBRA 120) at 80 kV. The images were analyzed using ImageJ 1.48 software to assess the morphology of *G. xylinus*.

Film thickness and uniformity

The film thickness of native purified BC membranes (65 mm x 65 mm) was measured using a digital caliper (WIHA 29422). Five measurements were taken for each sample at various random locations. The uniformity was determined as standard deviation (SD) of the 5 measurements. All the measurements were done in triplicate and the results were expressed as mean $\pm$ SD.

Film weight and yield

The weight of native BC, BCd and BCm (32.5 x 32.5 mm) was accurately measured using an analytical balance (KERN ABT 100-5NM). The dry mass was determined gravimetrically by oven-drying the samples at 40°C until constant weight (cca. 30-60 min) (Alonso et al. 2018). Then the water content and BC yield were computed (Castro et al. 2012; Hornung et al. 2006). All the measurements were done in triplicate and the results were expressed as mean $\pm$ SD.

OPTIMIZATION STUDY

Experimental design and target optimal levels for the response parameters

A Box-Behnken Design (BBD) experimental design (Ahmed et al. 2019; Bae and Shoda 2005; Du et al. 2018; Tan et al. 2012) was used to optimize the fermentation conditions to obtain BC with properties fit for biomedical purposes: drug release (Y_1) (Ullah et al. 2017), mechanical properties (Y_2) (Li et al. 2015b), structure (Y_3) (Wei et al. 2011), as response variables. Two independent variables were considered at 3 levels: inoculum volume (X_1) at 1 mL; 3 mL; and 5 mL; and fermentation period (X_2) at 6 d; 12 d; and 18 d. The levels were selected based on the results from our preliminary study, but also, according to current research (Bilgi et al. 2016; Du et al. 2018; Rangaswamy et al. 2015; Zeng et al. 2011). The BBD generated 15 experimental runs for the fermentation conditions (represented by X_1 and X_2) needed to obtain the target response variables (Y_1 , Y_2 , Y_3) for the Response surface methodology (RSM) 3 replicates

were set at the center of the design for the estimation of the pure error sum of squares (Ferreira et al. 2007; Lim et al. 2019). The experimental runs were randomized and carried out in unblocked design (Table 2).

The following levels for each of the response variables were targeted for the multi-response analysis in the optimization procedure: a target thickness of 2 mm (Fu et al. 2012), highest half-swelling time (Campano et al. 2015), highest drug half-release time, highest tensile strength and minimum Young's Modulus (Amin et al. 2012; Campano et al. 2015; Lin et al. 2013) and minimum fiber diameter (Fu et al. 2013). The target thickness of 2 mm was chosen for optimal properties, as recommended by Fu et al. (2012). The highest possible value was chosen for half-swelling time (Campano et al. 2015; Li et al. 2015a), drug half-release time (Amin et al. 2012; Reiniati et al. 2017), and tensile strength (Amin et al. 2012; Campano et al. 2015; Lin et al. 2013) to obtain BC with the best uptake and release properties, but with a good tensile strength. However, the minimum Young's Modulus (Amin et al. 2012; Campano et al. 2015; Lin et al. 2013) and minimum fiber diameter (Fu et al. 2013) were chosen to obtain an elastic BC that can be properly manipulated, and with a 3D structure that would retain and release optimally any incorporated substance.

Fourier transform infrared spectroscopy (FTIR)

The infrared absorption spectrum for each sample was recorded using the Shimadzu IRPrestige-21 spectrophotometer (Alonso et al. 2018; Gea et al. 2011; Zhang et al. 2011). Because of the structure of BC, the sample was applied directly to the ATR (attenuated total reflectance) horizontal diamond accessory with a single reflection from PIKE and pressed using its Hight Force Clamp. The spectrum was recorded at a wavelength range of 600-4000 cm^{-1} , with a resolution of 4 cm^{-1} with 16 scans per measurement, according with previous studies (Terzjak et al. 2015). Cellulose from filter paper was used as a reference for the functional groups in BC. The primary data obtained were processed using the IR solution Software programs Overview (Shimadzu) and OriginR 7SR1 Software (OriginLab Corporation, Northampton, USA).

Water uptake ability: swelling ratio, and moisture content

The initial weights of BCd and BCm (32.5 x 16.25 mm, prepared as described before) were accurately measured. Then each sample was immersed in a vial containing 20 mL of deionized water and incubated at room temperature (Lin et al. 2013). At precise intervals (10 min; 20 min; 30 min; 1 h; 6 h and 24 h) the swollen membranes were weighted after the removal of excess surface water by gently tapping with filter paper. The swelling behavior and moisture content of the membranes were determined using equations (1) and (2).

$$\text{Sr (\%)} = (W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}} \cdot 100 \quad (1)$$

$$\text{Mc (\%)} = (W_{\text{wet}} - W_{\text{dry}}) / W_{\text{wet}} \cdot 100 \quad (2)$$

where: Sr = swelling ratio; Mc = moisture content ratio; W_{wet} = weight of swollen pellicles; W_{dry} = initial weight

The half-swelling time was obtained by plotting the Sr by time and computing the duration in which the pellicles swell at the half of their W_{wet} (Li et al. 2011). All the measurements and computations were done in triplicate and the results were expressed as mean $\pm$ SD.

Drug release and drug half-release time

The kinetics of drug release was estimated using beet juice, as immersion fluid (Basak et al. 2015). Firstly, a standard curve of beet juice concentration versus absorbance at 520 nm (Shimadzu UV-1900) (Basak et al. 2015) was obtained by measuring various dilutions (0.1%; 0.3%; 0.5%; 0.7%; 1%; 1.5%). The obtained curve had the following equation: $y = 0.3644x + 0.0007$ and an R^2 of 0.9996.

For the actual drug release assay, BCd and BCm (32.5 x 16.25 mm) membranes were loaded with concentrated beet juice overnight. The membranes thus prepared were immersed in 20 mL distilled water and a 2 mL solution was taken at precise time intervals (30 min; 1h; 2h; 3h; 6h; 24h; 48h; 72h) and their absorbance was measured at 520 nm (Shimadzu UV-1900) to determine the beet juice release (Basak et al. 2015). After removing aliquots of 2 mL from each sample for analysis, the same volume of fresh distilled water was added (Wu et al. 2014). UV absorbance was measured to determine the concentration of released beet juice at each time point (Yoshino et al. 2013). The drug half-release time was obtained by plotting the released beet juice by time and computing the duration in which the pellicles release half of their total uptake (Li et al. 2011). All the measurements and computations were done in triplicate and the results were expressed as mean $\pm$ SD.

Mechanical properties

The mechanical properties of the BCd and BCm samples were determined by standard tensile tests. All measurements were carried out at room temperature (23°C) and humidity in the range of 45–50%. Specimens of 6.5 cm in length, 2 cm in width, and 0.6 mm thick were loaded to failure with constant crosshead speed (2 or 4 mm/min) using an tensile test machine (Instron 3366 (10 kN) (Paşcalău et al. 2012). Five specimens were tested for each BCm and BCd sample. The maximum load (N), tensile strength (MPa), elongation at break (%), Young's modulus (MPa), and stiffness (kN/cm) were calculated. The results were reported as mean $\pm$ SD of 5 measurements.

Scanning electron microscopy (SEM)

Prior to SEM analysis the BCm were treated with 1.6% glutaraldehyde in a sodium cacodylate trihydrate ($\text{C}_2\text{H}_{12}\text{AsNaO}_5$) buffer (0.1 M, pH 7.4) for 1 h; afterwards each sample was washed 3-5 times every 5-10 min with the $\text{C}_2\text{H}_{12}\text{AsNaO}_5$ buffer, and then left in the buffer solution for 1 d. The next day the samples were lyophilized in a Critical Point drier. BCd and processed BCm membranes were sprayed with Au and Pd (80:20 ratio) in a sputtering apparatus (Leica EM ACE600). All prepared samples were prepared in duplicate and analyzed with ZEISS EVO electronic microscope (Alonso et al. 2018; Kirdponpattara

et al. 2015; Santoso et al. 2020). Image analysis and fiber diameter measurement were performed with ImageJ 1.48 software. The diameters were analyzed in 5 different images fields per each sample measuring the diameter of minimum 100 fibers per field (Kirdponpattara et al. 2015).

Statistical analysis, Response Surface Methodology (MRS) and model fitting

XLSTAT (version 2020.4.1) and Minitab® (version 19.2020.1) statistical software programs were used to analyze the data. A one-way ANOVA ($p < 0.05$) within samples was used to compare the effects of inoculum, harvest day, and processing type. Fisher pairwise comparisons (LSD, $P = 0.05$) was applied whenever ANOVA indicated significant differences among the samples. Pearson correlation coefficients were computed. Linear regression analysis was used to quantify the effect of harvest day, inoculum volume, and processing with a confidence interval of 95%, a tolerance of 0.0001 and a model selection based on best model by adjusted R^2 .

Correlational Principal Component Analysis (PCA) was performed on the preliminary data formatted in observations/variables table (Cătușescu et al. 2019). Three of the 5 identified factors (components) were selected: F1 which had an Eigenvalue of 2.67 and accounted for a variability 53.48%, F2 which had an Eigenvalue of 1.11 and accounted for a variability 22.15%, and F3 which had an Eigenvalue of 0.72 and accounted for a variability 14.45%.

Response Surface Methodology (RSM) was used on a fully factorial design with 5 levels for the preliminary study and on a Box-Behnken experimental design for the optimization procedure (Ahmed et al. 2019; Bae and Shoda 2005; Du et al. 2018; Tan et al. 2012). For the preliminary study 2 continuous explanatory variables (X_1 : harvest day, X_2 : inoculum volume) were chosen and 1 response variables variable (Y_1 : thickness). For the optimization study the 2 continuous explanatory variables (X_1 : harvest day, X_2 : inoculum volume) were kept and 1 categorical variable was added, X_3 : type of membrane. Their affect was modelled on 6 response variables (Y_1 : thickness; Y_2 : half-swelling time; Y_3 : drug half-release time; Y_4 : tensile strength; Y_5 : Young's modulus; Y_6 : fiber diameter). Linear, interaction, and squared coefficients of the model were determined by least square regression. A stepwise approach was followed to select the terms in the mathematical model and an $\alpha \leq 0.15$ was set for a term to enter the model. Additional terms were added in the final step to maintain the hierarchical model. 2D response surface charts and the desirability functions were also computed. ANOVA and Mann-Whitney two-tailed test were performed to analyze the fitting of the model. For response optimization the following goals were set: thickness – target to 2 mm; fiber diameter – minimum; swelling half-life – maximum; drug-release half-life – maximum; tensile strength – maximum; Young's modulus – minimum. For the validation of the models, new experimental runs were compared with the PI 95% of each theoretical value of the response parameters.

Results And Discussion

Preliminary study

Transmission electron microscopy TEM

Both purified and unpurified pristine BC membranes were analyzed to assess the effectiveness of NaOH and NaOCl purification treatment. In pristine BC, the bacteria cells occurred randomly in the membrane structure. The bacteria had ellipsoidal to rod shapes with dimensions between 0.3-0.7 x 0.8-1.4 μm (Figure 1. a, b) similar to current reports (Campano et al. 2015). The bacteria wall was between 0.02 and 0.03 μm (Figure 1. c). This confirmed that the *G. xylinus* cultures were not infected during the handling and fermentation with other species.

Purification is a crucial step in the production of any cellulose product, and more so when aiming a biomedical application. This process is intended to remove all non-cellulose materials such as proteins and nucleic acids derived from bacterial cells and the culture medium. Additionally, the purification process allows the formation of strong inter- and intra- fibrillar hydrogen bonds (Gea et al. 2011). The most widely used purification procedure is the treatment of the harvested pellicles with 0.1 M NaOH at 60 - 80°C for 1 - 3 h; then repeatedly washings with distilled water until reaching a neutral pH (Alonso et al. 2018; Gea et al. 2011; Wang et al. 2017). The proper purification of BC is important because molds usually start to grow within a few weeks in improperly purified BC membranes, which become darker and opaque (Gea et al. 2011).

In our case, the pristine BC films were yellow when harvested from the culture medium and became brownish after the NaOH treatment, with random transparent areas. However, after a 24-hour NaOCl treatment, the BC membranes reached transparency, showing the desired gel-like structure. TEM imaging confirmed the effectiveness of the treatment, as no rod-shaped bacteria nor other impurities were present in the internal structure of the membrane, even after 6 months. Thus, the two-step treatment that we proposed purified optimally the BC and stopped mold growing. This ensured longer storage, up to 6 months, without experiencing any change in color and/or quality.

Film thickness, uniformity

BC samples had different thickness according to the inoculum volume and harvest day (Supplementary data Table 2). The thickness varied for all pellicles from 1.34±0.20 mm in the 6th harvest day, to 2.6 7±0.67 mm in the 18th day. It was also observed that all samples inoculated with 1 mL were thicker than other samples, however, the difference was significant only in the 9th day of harvest.

The thickness of the films was significantly influenced by both the inoculum volume ($p < 0.008$) and the harvest day ($p < 0.0001$), as resulted from linear regression analysis. However, the duration of fermentation was the most influential factor. Thus, the inoculum volume was removed from the regression analysis, as suggested, and a linear equation was obtained: thickness (mm) = 1.19 + 6.56 • 10⁻² • day. Although the model was statistically confirmed, only 47% of the variability was explained by the parameter day ($R^2 = 0.47$). Thus, a further response surface regression of thickness as response variable was performed on the factorial design with 5 levels (Figure 2) and the model presented in Table 1 was generated.

Table 1. Coded coefficients of the response surface regression of thickness (Y_1) performed on the factorial design with 5 levels and regression equation in uncoded units

Model/term	linear	b0	b1	b2	square	b11	b22	lack-of-fit	R^2
coefficient		1.966	0.393	-0.047		-0.130	0.167	0.431	0.50
P-value	0.000	0.000	0.000	0.354	0.054	0.143	0.053		
Regression equation:	$Y_1 = 1.123 + 0.149 \cdot X_1 - 0.274 \cdot X_2 - 0.004 \cdot X_1^2 + 0.042 \cdot X_2^2$								

Note: The explanatory variables were X_1 : harvest (d) and X_2 : inoculum volume (mL). A stepwise selection of terms was used with $\alpha \leq 0.15$ for a hierarchical model.

Rangaswamy et al. (2015) concluded that the inoculum volume plays an important role in BC production. Similar to our study, the results showed that BC production increases with the inoculum volume. However, this seems to be true until the inoculum volume reaches the threshold of 5% (v/v). Higher percentages generated a decrease in cellulose production (Rangaswamy et al. 2015) although cellulose production was observed in all inoculum size tested, values lower or higher than 5% inoculum showed a decrease in cellulose production. In contrast, other studies concluded that the total cell count is not significant for BC production (Hornung et al. 2006) because the really significant quantity is the number of bacteria within the aerobic zone, which are producing the cellulose. Thus, based on our results and previous reports we kept the inoculum volume in our experiment design for the optimization study.

The uniformity was not influenced by inoculum or fermentation period; however, there was a significant difference between the samples ($p = 0.033$), as seen in Supplementary data Table 2. Additionally, thickness was negatively correlated with the uniformity ($r = -0.595$; $p < 0.05$) in Pearson correlation analysis (Supplementary data Figure 1 and Table 1). Thus, the thicker the membrane, the more uniform tends to be.

Similar to our results, some studies showed that the thickness may vary from 0.01 mm at 48 h incubation period (Machado et al. 2016) to 8 mm after 2 weeks (R. Rebelo et al. 2018) in a static culture. Also, Blanco Parte et al. (2020) reported that the thickness of the BC becomes unevenly during the fermentation period. However, other studies reported no apparent variations or obvious imperfections after direct visual evaluation of BC membranes (Sokolnicki et al. 2006). Nonetheless, the thickness of BC films may be controlled by adjusting the incubation period and improving the fermentation method (Fu et al. 2012).

Film weight and yield

An increase in both dry weight and water content was observed with increasing fermentation period (Supplementary data Table 2). The dry weight varied from a minimum of 2.63 ± 1.10 mg to 9.83 ± 1.46 mg, that is approximately from 0.3% to 0.9%, while the yield varied from 0.11 ± 0.04 g/L to 0.39 ± 0.06 g/L. BC yield was the lowest in all 1 mL inoculum samples. The productivity in BC increased from the 6th harvest day (0.11 ± 0.04 g/L) to the 15th day (0.27 ± 0.15 g/L). The 15th fermentation day had the highest cellulose yields and the results are in accord with current studies (Castro et al. 2012; Hornung et al. 2006). Hornung et al. (2006) reported that BC yield increased with fermentation time, cellulose formation ceased, however, after 15 fermentation days. Castro et al. (2012) observed that even though BC yield increased from 2nd to 8th incubation day, BC production decreased between the 8th and 14th fermentation day.

Our results are in line with current results which state that the content of BC in initial state being no higher than 1% (Castro et al. 2012; Rangaswamy et al. 2015; Skvortsova et al. 2019). BC is a hydrogel-like membrane, and its fibrous and network-like structure contribute to its very high water content (Gea et al. 2011). This property is important when considering its biomedical application as wound dressing material (Bodea et al. 2019; Fu et al. 2013).

The regression analysis showed that the harvest period significantly influenced BC dry weight ($p < 0.002$), water content ($p < 0.0001$), and yield ($p = 0.002$), while the inoculum volume seemed to have no significant influence.

Optimization study

For the optimization study a BBD experimental design was used to obtain the variable combinations for the experimental runs (Table 2). This enabled the experimental runs of a smaller number of samples with reliable results because the number of levels for the factors is minimized (from 5 to 3 in our case) (Pal and Jadeja 2019; Rodsamran and Sothornvit 2019) and it generates less extreme experimental combinations compared to Central Composite Design (CCD) (Otto 2016). The prediction precision around the supposed optimum is similar to the CCD because the center point level is repeated (3 times in the current study), but with fewer runs (Addinsoft 2020). However, the efficiency of BBD was shown to be higher than CCD and Three-Level Full Factorial (Ferreira et al. 2007; Pal and Jadeja 2019), providing a model with a better fit. Thus, by using the BBD the number of experiments was significantly reduced compared to the fully factorial design with 5 levels as in the preliminary study. In addition, less extreme experimental combinations were tested.

Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) analyzes cellulose using the chemical bonding present in the biopolymer. It is an alternative for qualitative analysis and it enables the identification of BC types and their purity (Alonso et al. 2018; Gea et al. 2011; Nishi et al. 1990; Watanabe et al. 1998).

In this study, the functional groups of all 5 variants of BC were confirmed by comparison with the infrared spectrum of filter paper (FP). As shown in Figure 3, the bacteria *G. xylinus* produced BC containing 20 identified functional groups, the majority similar to FP. The adsorption at 3332 cm^{-1} present in FP spectra could be attributed to stretching vibration of intra and inter O-H bond in cellulose (Yassine et al. 2016). This absorbance peak is present in all BC samples, but its position seemed to vary slightly with the volume on inoculum. The peak was positioned at lower wavenumbers for samples with 5 mL inoculum and higher wavenumbers for samples with 1 mL, probably because of the variations in the BC structure.

The 2899 cm^{-1} peak could be attributed to C-C stretching of CH_2 and CH_3 groups or CH_2 asymmetric stretching (Tercjak et al. 2015). The FP spectra shows a peak at 1647 cm^{-1} corresponding to H-O-H bending of absorbed water (Yassine et al. 2016). In BC samples this peak appears shifted to lower wavenumbers:

1645 cm^{-1} to 1639 cm^{-1} . The peak at 1427 cm^{-1} present in all samples may correspond to CH_2 scissoring (Dammström et al. 2005), but most studies attribute it to CH_2 symmetric bonding or O-H in plane bending (Ashori et al. 2012; Barud et al. 2011). The peak at 1365 cm^{-1} in FP spectra could be assigned to C-H bending. This peak was shifted to lower wavenumbers in all BC samples, similar to other peaks. The peak at 1334 cm^{-1} could correspond to C-H deformation or O-H in-plane bending (Castro et al. 2011) and absorption at 1315 cm^{-1} may be assigned to out-of-plane wagging of the CH_2 groups. The absorption at 1159 cm^{-1} is observed in FP and all BC spectra, with a shift to higher wavenumber (1161 cm^{-1}) for samples with 1 mL inoculum. This is a typical indicator of the presence of C-O-C antisymmetric bridge stretching of 1,4-b-O-glucoside in BC (Amin et al. 2012).

It was shown that the peaks around 1000-1100 cm^{-1} can be assigned to C-O stretching vibrations in primary alcohol and C-O-C skeletal vibrations (Barud et al. 2011), but this hypothesis is controversial. For example, some studies attributed the absorption at 1030 cm^{-1} and 1054 cm^{-1} to the bending of C-O-H bond of carbohydrates (Huang et al. 2015; Sun et al. 2010) or C-O-C pyranose ring skeletal vibration (Castro et al. 2014; Yassine et al. 2016). These peaks are also present in all the studied samples. Gao et al. (2011) reported that the peak at 1029 cm^{-1} (present in FP and all BC samples spectra) might be also associated with the presence of OCH_3 , while the peak at 1107 cm^{-1} (present in FP and all BC samples spectra) indicated C-C bonds of the monomer units of polysaccharides or C-O bending vibration (Castro et al. 2011). An intense peak was observed at 1003 cm^{-1} in the BC spectra, originated from the stretching vibrations of $\text{C}_3\text{-O}_3$, which was the main bonding forming a cross-linking structure (Garside and Wyeth 2003).

The peak at 896 cm^{-1} present in FP and all BC samples spectra could be assigned to antisymmetric out-of-phase ring stretching of beta-glycosidic linkages between the glucose units, which is designated as an amorphous absorption band (Dayal et al. 2013; Goh et al. 2012).

In conclusion, BC produced by *G. xylinus* seems to consist mainly of pure cellulose I because of the 2 weak peaks at around 1427 cm^{-1} and 898 cm^{-1} and other peaks corresponding to pure cellulose (Goh et al. 2012; Nelson and O'Connor 1964). Additionally, we found peaks at around 3338 cm^{-1} , 1160 cm^{-1} , and 900 cm^{-1} , all well studied previously and attributed to cellulose I (Rani et al. 2011a; Rani et al. 2011b). The peaks observed at 1334 cm^{-1} , 1315 cm^{-1} , 1278 cm^{-1} and 1427 cm^{-1} show, however, evidence of the presence of cellulose II as well (Rani et al. 2011a; Rani et al. 2011b).

Water uptake ability: swelling ratio and moisture content

The BCd and BCm batches took up significant amounts of water during the 24 h (Figure 4, Supplementary data Table 3), both showing significant different swelling ratios at the end compared with the beginning of the trials ($p < 0.0001$, $p = 0.0004$, respectively). The swelling activity among BCd samples was statistically different only up to the first 10 min ($p = 0.046$). The samples obtained with 5 mL inoculum and harvested after 18 d had the highest swelling ratio after 10 min (1079.41 $\pm$ 81.60%) (Figure 4 b), while the membrane with 3 mL harvested after 12 d, which had the lowest (694.79 $\pm$ 66.50%). The swelling activity up to 10 min was significantly influenced by the volume of inoculum used to obtain the BC ($p = 0.039$), while the harvest day seemed to have no significant individual influence. Li et al. (2015b) showed that the water uptake ability of moist BC after 10 minutes was 44 times its own weight, thus higher than in the present study. The swelling increased gradually and reached 52 times its own weight after 12 h, while in our case it reached only 1792%.

Both types of BC membranes swelled rapidly in the first half an hour. After this moment, BCm increased gradually in the next 6 h, then it maintained its swollen mass (Figure 4 A). On the other hand, BCd increased gradually up to 24 h (Figure 4 b). Similarly, Lin et al. (2013) showed that moist BC membranes swelled gradually for 6 h, then maintained a stable state for the next 60 h. Unlike our study, Rambo et al. (2008) observed that oven-dried BC reached the maximum swelling ratio (175%) after 30 min, then decreased slightly to 153% between 0.5 and 1 h, and achieved a stable state after 1 h.

The swelling ratio over time of the BCd samples taken as a whole was significantly influenced by all 3 tested inoculum volumes, with the pellicle obtained with 5 mL achieving the highest swelling ratio (2121.73%). On the other hand, only the harvest days 12 and 18 significantly influenced the swelling ration of BCd samples taken as a whole. Six days of fermentation probably generated pellicles too thin with loose fibrillar network. In contrast, all BCm samples had comparable swelling ratios at every tested interval, thus no influence of the 2 fermentation variables was observed. However, when considering BCm as a batch, a significant influence of the swelling ratio over time by the inoculum volume was observed for the samples obtained with 5 mL ($p < 0.0001$). Similarly, only a fermentation period of 12 days seems to significantly influence this relation.

The BCd batch, considered as a whole, showed significant differences in swelling rations up until 60 min after soaking, while BCm until 20 min ($p < 0.001$). Thus, BCm samples swelled significantly faster than BCd ($b_m = 22.07$ vs. $b_d = 32.52$), suggesting that the oven-drying processing affects the swelling ratio of BC by probably modifying the morphology of the pellicle network (Fu et al. 2013; Lin et al. 2013; Zhang et al. 2011). Thus, the oven-drying processing negatively affects the uptake speed of the BC membranes. Similar to our results, Sanchavanakit et al. (2006) reported that BC membranes in a wet state had a swelling ratio up to 10 times higher (1027%), than air-dried BC (106%). Wu et al. (2018) observed that BC membranes in wet state showed a high swelling ratio of 169% due to its fibrous network structure. Additionally, Alonso et al. (2018) observed that the rehydration ability of freeze dried BC is far superior (up to 9 times) compared to oven-dried BC, which had a much lower swelling ratio capacity (50%). This could be due to pore preservation during manipulation, compared to oven-dried pellicles, which altered pore structure. The compact structure, due to drying, reduced the swelling ability. Thus, the processing techniques altered the BC pore availability and reduced the swelling ability of dried BC. In contrast, our results showed that BCd had the highest swelling ratio after 24 h (2121%) in the samples with 5 ml inoculum harvest in the 18th day, compared to the same BCm samples (1600%). This may be due to our reporting of the swelling ratios of both BCm and BCd to their initial weight and not to the initial dry mass.

Both tested fermentation variables, the inoculum volume, and the harvest day, significantly influenced the swelling activity BC, as a whole, ($p < 0.002$). In general, a volume of inoculum of 1 mL produced pellicles with significant swelling up to a mean of 20 min, while higher volumes up to 30 min. This shows, once again, that uptake ability of BC is influenced by the inoculum volume, most probably because of the difference in thickness of the pellicles (Supplementary data Table 2), their difference in network morphology, and fiber thickness. The thickness varied for all pellicles, according to the inoculum

volume and harvest day, from 1.34 ± 0.20 mm in the 6th harvest day, to 2.67 ± 0.67 mm in the 18th day (Supplementary data Table 2). Yoshino et al. (2013) reported that BC expanded up to 80% in the first 10 min and about 3 times its initial thickness after 4 h, reaching to a thickness of 2.5 mm. Juncu et al. (2016) compared different films of sodium carboxymethyl cellulose with increasing BC content. The study revealed that the swelling degree decreased with the increase of BC content. A content of 12.5 mg of BC in the membranes showed the highest swelling ratio. In our case, the swelling ratio increased progressively and constant during the 24 h, probably because our maximum cellulose content was 9.83 ± 1.46 mg, thus below the 12.5 inflection point.

BC mechanical properties were in indirect correlation with the swelling ratio. The maximum load, tensile strength ($r = -0.534$; $p = 0.002$), Young's Modulus and stiffness ($r = -0.506$; $r = 0.004$) were negatively correlated with the swelling ability of the BC membranes. In contrast, the elongation at break is positively correlated to the uptake ability ($r = 0.534$; $p = 0.002$). A reduced water content in BC membranes brings the fibers closer together, which gives the tendency to assemble with each other into more compact fiber bundles leading to a more compact structure (Fu et al. 2013; Zhang et al. 2011). The uptaken water in BC pellicles limits the interactions among the fibers by competitive water–cellulose hydrogen bonds. Water in BC pellicle is crucial to achieving a good alignment of the BC fibers, and thus good mechanical properties since water can serve as a plasticizer (Wang et al. 2017).

The half-swelling time of BCd varied between 0.99 ± 0.38 h and 2.12 ± 0.84 h, with significant differences among samples (Table 2). The fermentation had an impact, with samples harvested after 18 days having higher half-swelling times. For the BCm this variation was between 2.47 ± 0.20 h and 2.68 ± 0.18 h, but with no significant differences. Linear regression analysis showed that only significant influence on the half-swelling time of all BC samples seems to be the processing. Oven processing affected negatively the half-swelling time ($b = -1.028$), with a mean value of 1.5 h compare to 2.52 h for BCm.

An ideal wound dressing must have a high water uptake ability, to avoid the accumulation of wound exudate, which reduces the wound healing, and permit the oxygenation to the wound (Li et al. 2015b; Wei et al. 2011; Wu et al. 2018). Thus, the swelling ratio and half-swelling time are important parameters for BC intended as wound dressing. BC would need to absorb the exudate and maintain proper wound moisture during the healing process, which promotes the penetration of active substances embedded in the dressing, and provide a painless removal after recovery (Lin et al. 2013). Additionally, a high uptake ability is also needed for a dressing delivering controlled-release active substances (Juncu et al. 2016; Wei et al. 2011). These results are in accord with previous studies, Wei et al. (2011) suggested that the highly porous structure permitted loading of drugs into the hydrogel fibrillar matrix. BC has shown to possess this ability, being thus suited for this biomedical use.

The moisture content of both BC membranes increased at the end compared with the beginning of the trials (Figure 5, Supplementary data Table 4). BCm and BCd moisture content was significantly different after 10 min and 24 h ($p = 0.013$; $p = 0.033$, respectively). The water content of BCd increased from 90% after 10 min to 95% in 24 h, compared to BCm, with a moisture content of 86% after 10 min and reaching 93% after 24 h. It was observed that the oven-drying processing significantly affected the moisture content of BC membranes ($p = 0.012$). BCm had a loosely interconnected network structure, compared to BCd (5.2.6. Scanning electron microscopy (SEM)). Yet, the structure of oven-dried BC was more compact with no visible pores on the surface, as described by Ullah et al. (2017), which explains the much lower moisture content of BCd compared to BCm. Lin et al. (2013) showed similarly to our study that the moisture content of moist BC membranes increased gradually until 6 h, than they maintained a stable state for the next 60 h.

The diameter of the fibrils in the BC membranes is in direct correlation with the moisture content ($r = 0.431$; $p = 0.018$). The matrix of BC membranes consists of randomly arranged fibrils and a variety of empty space in-between which helps absorb and store water inside the membrane (Lin et al. 2013). There was a positive correlation between BC moisture content and Young's Modulus and stiffness ($r = 0.431$ and $r = 0.432$; $p = 0.017$; respectively). Also, the moisture content is negatively correlated to the elongation at break ($r = -0.454$; $p = 0.012$). The results are consistent other authors reporting that the mechanical behavior of BC essentially depends on its moisture content (Skvortsova et al. 2019).

BCd samples were significantly different after 10 min ($p = 0.060$). The pellicles obtained with 3 mL harvested after 12 days had the lowest moisture content (87%), while the membranes with 5 mL inoculum harvested after 18 days had the lowest moisture (91%) (Figure 5 b). The moisture content over time of the BCd samples taken as a whole was significantly influenced by both tested variables, with the pellicle obtained with 1 mL harvested in the 6th day up-taking the highest moisture content of 96% (Figure 5 b). Similarly, BCm samples taken as a whole were significantly influenced by all 3 tested inoculum volumes with the pellicles obtained with 5 mL ($p < 0.0001$) up-taking the highest moisture content (94%). On the other hand, only the harvest days 6 and 12 significantly influenced their moisture content. A fermentation period of 18 d probably generated pellicles too thick with a too dense fibrillar network (Figure 5 a).

The half-moisture time of BCd and BCm membranes was significantly different ($p = 0.010$) (Table 3) and linear regression analysis showed that processing might be the only significant variables influencing the half-moisture time of all BC samples. Oven-drying processing affected the half-moisture time ($b = 0.726$) with a mean value of 1.15 h compared to 0.43 h for BCm. The half-moisture time of BCm membranes was in direct correlation with the half-swelling time ($r = 0.844$; $p < 0.0001$), because, logically, the more a pellicle swells, the higher its water content becomes.

Drug release

The BCd and BCm membranes released significant amounts uptaken fluid (beet juice) during the 72 h (Figure 6, Supplementary data Table 5). Both BCd and BCm membranes showed a significant drug release capacity over time ($p < 0.001$). The release capacity was significantly influenced by all 3 inoculum volumes in both BCd ($p < 0.005$) and BCm ($p < 0.046$). The BCd samples obtained with 5 mL inoculum and harvested after 18 d had the highest drug release capacity (Figure 6), while BCm, had the highest drug release obtained for pellicles obtained with 3 mL inoculum and fermented for 12 (Figure 6). A gradual drug release was observed throughout the 72 h, with a significant difference between the 2 types of membranes throughout the tested period ($p < 0.0001$) (Figure 6). BCm samples had up to 3 times higher drug release capacity, than BCd ($b_m = -0.006$ vs. $b_d = -0.003$), suggesting that the oven-drying processing negatively affected the drug release ability of BC. The membrane type (moist or dry) significantly influenced the release capacity of BC ($p < 0.0001$), thus BCm had an almost 3 times higher drug release capacity than BCd (Figure 6). It seems that oven-drying makes the structure of BC more rigid and less porous (Alonso et al. 2018; Fu et al. 2013; Zhang et al. 2011). The three-dimensional porous structure of BCm can facilitate the drug release activity because of the

larger surface area that facilitates drug uptake and has faster release rates (Wei et al. 2011). Skvortsova et al. (2019) found that drying leads to distortions of the BC structure due to aggregation of cellulose fibrils. As a result, the porosity appears to decrease significantly. Contrary, Ullah et al. (2017) reported almost similar drug release rates for both the freeze-dried and oven-dried BC with no effect of the drying method. Regardless of the processing method, almost 100% of the drug was released within 45 min. The immediate drug release is linked to the hydrophilic nature and swelling behavior of BC. Wei et al. (2011) observed a stable and continuous release of the antimicrobial agent within 24 h.

Table 2. The properties of bacterial cellulose (BC) of interest for biomedical purposes at different fermentation conditions based on a Box-Behnken design for response surface methodology (RSM).

Independent variables				Response - dependent variables									
X ₁	X ₂	X ₃	Y ₁	Y ₂		Y ₃		Y ₄		Y ₅			
harvest (d)	inoculum volume (mL)	BC type	thickness* (mm)	half-swelling time (h)		drug half-release time (h)		Tensile strenght σ (MPa)		Young's Modu (MPa)			
			exp	pred**	exp	pred**	exp	pred**	exp	pred**	exp		
1	6	1	dry	1.68±0.16 ^{bc}	1.62	1.25±0.5 ^{bc}	1.11	4.95±0.77 ^{de}	5.63	7.61±0.21 ^{ab}	7.88	128.92±30.37 ^b	
2	18	1	dry	2.67±0.67 ^{ab}	2.81	1.92±0.89 ^{ab}	1.89	3.68±0.32 ^e	5.36	10.34±3.69 ^a	9.85	139.34±22.35 ^b	
3	12	3	dry	2.09±0.15 ^{ab}	1.93	0.99±0.38 ^c	1.50	12.78±3.45 ^a	10.28	10.04±1.90 ^a	8.86	117.86±28.18 ^b	
4	6	5	dry	1.34±0.15 ^c	1.05	1.22±0.6 ^{bc}	1.11	9.12±1.60 ^b	8.88	7.08±2.78 ^{abc}	7.88	143.99±36.54 ^a	
5	18	5	dry	2.28±0.23 ^{ab}	2.23	2.12±0.84 ^a	1.89	8.25±1.61 ^{bcd}	8.61	9.22±3.33 ^a	9.85	209.39±23.85 ^c	
6	6	1	moist	1.68±0.16 ^{bc}	1.62	2.47±0.20 ^a	2.52	5.93±0.58 ^{bcd}	3.81	3.02±0.64 ^d	2.45	16.03±2.97 ^c	
7	18	1	moist	2.67±0.67 ^{ab}	2.81	2.53±0.28 ^a	2.54	3.77±1.76 ^e	3.54	4.64±0.32 ^{bcd}	4.42	26.38±15.22 ^c	
8	12	3	moist	2.09±0.15 ^{ab}	1.93	2.68±0.18 ^a	2.53	5.97±2.25 ^{bcd}	8.46	2.91±0.83 ^d	3.44	21.59±11.90 ^c	
9	6	5	moist	1.34±0.15 ^c	1.05	2.49±0.38 ^a	2.52	5.38±1.95 ^{cde}	7.06	2.61±0.38 ^d	2.45	12.44±0.73 ^c	
10	18	5	moist	2.28±0.23 ^{ab}	2.23	2.47±0.08 ^a	2.54	8.60±2.81 ^{bc}	6.79	4.00±0.55 ^{cd}	4.42	21.82±2.47 ^c	
p-value***				0.992		0.824		0.853		0.897		0.971	

Where: exp – experimental values; pred – values predicted by the RSM model; desir – overall desirability (0...1); * - the thickness was measured for the entire batch, before drying (n=6); **the predicted value resulted from the model optimizing the BC properties; ***Mann-Whitney two-tailed test ($\alpha = 0.001$) of the experimental data versus the values predicted by the model optimizing the BC properties

Note: The data are presented as mean $\pm$ SD. Different letters (a-h) within the same column show significant differences among the samples (Fisher (LSD), $p < 0.05$)

Generally, the drug release from hydrogels can be influenced by many factors like swelling, drug concentration, drug characteristics, and the hydrogel structure (Zeinali Kalkhoran et al. 2018). The highly porous structure of BCm (Supplementary data Figure 2) and BCd (Supplementary data Figure 3) can easily permit the loading of drugs into their matrix and subsequent drug release. It was observed that the samples obtained with 5 mL inoculum and harvested after 18 d had the highest swelling ratio in both BC membrane types, but BCm had a significantly better drug release behavior. BCd maximum drug release was obtained for the samples with 3 mL inoculum harvested in the 12th day. The rate of drug release depends on the water content of the swollen hydrogel, and on the structure of the fibrillar network (Wei et al. 2011). Juncu et al. (2016) stated that drug release decreased with the increase of BC content. Therefore, the concentration of BC could be an important factor which may control drug release. Additionally, they observed that the release ability presented a sudden growth during the first 20 min. The dry BC films released about 66% of the drug within one day (Wei et al. 2011).

Both tested variables, the fermentation period and the inoculum volume, significantly influenced the drug release of both BC membranes. The inoculum volume significantly influenced the drug release capacity of both membranes up to 48 h. The release capacity over time of the BCm samples taken as a whole was significantly influenced by all 3 tested fermentation periods, compared to BCd samples which were only influenced by the 6th and 12th days. BCd samples with 5 mL had significantly higher release ability. BCm release ability was also significantly influenced by 3 mL inoculum volume which had the higher drug release, up to 6 h (Figure 6), with the highest values in the 12th harvest day compared to the 6th day.

The half-release time of BCd varied between 3.68±0.32 h and 12.78±3.45 h, with significant differences among samples (Table 2). For the BCm this variation was between 3.78±1.76 h and 8.60±2.81 h, but with no significant differences. The inoculum volume affected the half-release time ($b = 0.813$, $p = 0.008$). An inoculum volume of 5 mL and 3 mL had a significant influence on half-release time of both membrane types ($p = 0.002$). The fermentation period only influenced the half-release time of BCd, with the highest value of 12.78±3.45 h.

BC is an attractive material for the fabrication of intelligent adsorptive materials for drug-delivery applications because of its properties: an ultrafine fibrous network structure, a good water-absorbance capacity, and optimal mechanical properties (Amin et al. 2012). BC appears to be a promising wound dressing material because of its ability to absorb exudate and controlled release of medical substances at the initial stage of wound healing porous (Alonso et al. 2018; Fu et al. 2013; Zhang et al. 2011).

Mechanical properties

Wound dressing materials need strength, flexibility and should maintain their integrity during use (Li et al. 2015b; Lin et al. 2017; Lin et al. 2013). BCd with 1 mL inoculum harvested in the 18th day had the maximum load of 12.41 N and a tensile strength of 10.343 MPa, compared to the BCm samples, with a less than half that resistance of only 5.571 N and 4.642 MPa respectively (Table 2, Table 3). The tensile strengths obtained in the current study for BCd is in agreement with previous studies were it ranged between 10.32 MPa (Fu et al. 2012) and 48.17 $\pm$ 15.38 MPa (Costa et al. 2019). And similarly, for BCm tensile strengths of 14.77 MPa (Lin et al. 2013) up to 207.0 MPa (Gea et al. 2011) were reported.

Among the BCd samples only the 5 mL inoculum harvested in the 18th day membrane was significantly different, and only for Young's modulus and the stiffness ($p = 0.022$). This sample had the highest values of 209.389 MPa and 41.865 kN/cm, respectively. In contrast, BCm samples significant higher values for maximum load and tensile strength ($p = 0.007$) were observed for the samples harvested in the 18th day.

Table 3. Characteristics of dry and moist bacterial cellulose membranes dependent on the harvest day and inoculum volume, mean±SD

BC type	Harvest (d)	Inoculum volume (mL)	Half moisture time (h)	Maximum load (N)	Elongation at break, ϵ (%)	Stiffness, k (kN/cm)
dry	6	1	2.04±2.04 ^a	9.13±0.25 ^{ab}	6.95±1.79 ^b	25.79±6.08 ^b
		5	0.87±0.53 ^{ab}	8.50±3.34 ^{abc}	4.92±0.46 ^b	28.80±7.30 ^b
	12	3	0.58±0.20 ^b	12.05±2.28 ^a	9.84±3.17 ^b	23.57±5.64 ^b
	18	1	1.06±0.69 ^{ab}	12.41±4.43 ^a	7.94±3.39 ^b	27.87±4.47 ^b
moist	6	5	1.23±0.57 ^{ab}	11.07±4.00 ^a	4.90±0.65 ^b	41.87±4.77 ^a
		1	0.42±0.11 ^b	3.63±0.77 ^d	18.77±1.04 ^a	3.20±0.59 ^c
	12	5	0.46±0.31 ^b	3.14±0.46 ^d	21.74±5.10 ^a	2.49±0.15 ^c
		3	0.47±0.08 ^b	3.49±0.99 ^d	16.11±1.36 ^a	4.32±2.38 ^c
	18	1	0.45±0.12 ^b	5.57±0.38 ^{bcd}	21.74±6.25 ^a	5.28±3.05 ^c
		5	0.33±0.03 ^b	4.80±0.66 ^{cd}	18.40±4.47 ^a	4.36±0.49 ^c

Note: The data are presented as mean ± SD. Different letters (a-d) within the same column show significant differences among the samples (Fisher (LSD), $p < 0.05$).

The results of mechanical testing showed that there was a statistically significant difference between BCd and BCm, taken as 2 batches ($p < 0.0001$) (Table 2, Table 3). The maximum load and tensile strength were significantly higher in BCd membranes compared to BCm ($p < 0.0001$). The Young's modulus and stiffness were significantly higher in BCd samples, compared to BCm (Table 2, Table 3). The BCd samples with 5 mL inoculum harvested in the 18th day had a Young's modulus of 209.389 MPa, which was almost 10 times higher than the corresponding BCm samples. Our results are in line with other studies that reported a range from 131 MPa (Fu et al. 2012) up to 458.38 MPa (Costa et al. 2019) for the Young's modulus of BCd and 0.76 MPa (Li et al. 2015b) to 72.3 MPa (Lin et al. 2017) for BCm.

BCm had significantly higher elongation at break values, up to twice higher than BCd (Table 3). The current report shows that the elongation at break of BCd may vary between $2.26 \pm 0.73\%$ (Almeida et al. 2014) and 9.00% (Fu et al. 2012), in contrast to BCm, that ranges between 4.54% (Pourjavaher et al. 2017) and can reach up to 32.17% (Lin et al. 2013). This makes BCd membranes more brittle. This was in accord with our visual observation that BCm was more elastic and malleable, compared to BCd which was inflexible, and hard to bend. Similarly Fu et al. (2013) stated that the dry BC film was more brittle than the moist BC film and as a result it broke easier in the tensile test. During elasticity testing, a decrease in strength occurs, leading to cracks in the BC membrane until it ruptures. Thus, the increase in crack propagation occurs very quickly in dry pellicles due to the lack of elasticity (Costa et al. 2019).

Nevertheless, the mechanical properties of BC may vary based on culturing and processing parameters, such as inoculum volume, culture time, medium, or post treatment (Lin et al. 2013). Our results corroborate this statement, as the maximum load and tensile strength of the BCm was significantly influenced by the harvest day ($p < 0.001$). In contrast, the inoculum volume did not appear to influence mechanical properties of BCm. In contrast, the properties of BCd did not seem to be influenced by the inoculum volume or harvest day. It is observed, however, that the inoculum had a great influence on Young's modulus and stiffness ($p < 0.051$). But although close, it did not reach statistical significance.

Scanning electron microscopy (SEM)

The surface morphology of the BCd and BCm membranes as seen by SEM is shown in Supplementary data Figure 2 and Figure 3. The structure is a well-organized, highly porous, three-dimensional fibrillar network, similar to previous reports (Alonso et al. 2018; Bodea et al. 2019; Pourjavaher et al. 2017; Wu et al. 2018). The matrix of BC membranes consists of randomly arranged nanofibers and empty spaces distributed randomly in-between. This network structure helps absorb and store water or water soluble compound inside the membrane (Lin et al. 2013; Wu et al. 2018).

The average fiber diameter of BCm ranged between 40.60 ± 4.99 nm up to 49.30 ± 4.18 nm (Table 2), in accord with current research (Fu et al. 2012). The largest median diameter was measured for membranes with 1 mL inoculum volume harvested in the 6th day (Supplementary data Figure 2 a), significantly different from the membranes harvested in the 18th day with 5 mL of inoculum (Supplementary data Figure 2 d). Both tested variables seemed to negatively influence the diameter of the BC fibrils, with the inoculum volume having a greater impact ($b = -0.91$; $p = 0.013$) than harvest day ($b = -0.42$; $p = 0.001$). Fu et al. (2012) reported the fiber diameters of thick BC (138.6 ± 37.6 nm) and thin BC (189.2 ± 57.9 nm) and similar to our results showed that the diameter of the fibers decreased as the thickness of the membrane and the incubation period increases.

The fiber diameter of BCd ranged, on average, between 51.34 ± 6.99 nm and 41.40 ± 3.87 nm (Table 2), which is consistent with previous studies. Pourjavaher et al. (2017) reported a fiber diameter around 45–70 nm, while Volova et al. (2018) obtained a range of 52 nm to 173 nm for dried BC (freeze dried and oven-dried). The median fiber diameter of BCd ranged between 41.40 nm in membranes with 1 mL inoculum volume harvested in the 18th day (Supplementary data Figure 3 c) to 51.33 nm in membranes with the same inoculum volume but harvested in the 6th day (Supplementary data Figure 3 a) (Figure 7). The latter diameter was significantly higher ($p < 0.0001$). For BCd only the harvest day had a significant negative influence ($b = -0.47$; $p = 0.014$) similar in impact with BCm.

As reported by Zeng et al. (2014) the drying method did not seem to influence the fibril diameter. This is surprising considering the differences in macroscopic structure between the two BC membranes types.

Optimization of bacterial cellulose (BC) by Response Surface Methodology (RSM)

The properties of the 2 types of BC obtained with varying harvest periods and inoculum volumes as set by a BBD experimental design were analyzed by RSM. The predicted values are shown in Table 2. The Mann-Whitney two-tailed test showed non-significant differences between the experimental and predicted

values at a significance level of 0.001. The multiple regression analysis of the RSM generated a system of polynomial equations that proved to be good statistical models for the experimental attributes of BC (Table 4). The lack-of-fit of all obtained models showed was not significant at levels higher than $p = 0.440$ (Table 4). This suggests that the proposed models fit the data.

The model equations within the tested intervals (X_1 : 6...18 d and X_2 : 1...5 mL) in uncoded values for the membranes are presented in Table 5, equations (3) – (14).

The RSM contour plots show the relationship between the 2 continuous predictors: X_1 : harvest day and X_2 : inoculum volume, and the 3 fitted response variables (Y_3 : drug-release half-life; Y_5 : Young's modulus; Y_6 : fiber diameter) for each of the 2 types of membranes (dry and moist) (Figure 8). By using the RSM we generated a desirability function that enabled us to determine the mathematical optimum of the 3 parameters taken into consideration, within the proposed ranges.

Table 4. Model parameters (coded coefficients), p values and goodness of fit statistics obtained by response surface methodology (RSM) for each of the 6 response variables (Y_i)

		Y_1 thickness (mm)		Y_2 half-swelling time (h)		Y_3 drug half- release time (h)		Y_4 Tensile strenght, σ (MPa)		Y_5 Young's Modulus E (MPa)		Y_6 fiber diameter (μ m)		Desirability
		coef	p	coef	p	coef	p	coef	p	coef	p	coef	p	coef
intercept	b0	1.926***	0.000	2.013***	0.000	9.370***	0.000	6.149***	0.000	69.720***	0.000	45.580***	0.000	0.553***
	b1	0.591***	0.002	0.203*	0.054	-0.135	0.793	0.985**	0.012	11.940**	0.025	-2.677***	0.000	0.066***
	b2	-0.289***	0.000	NA	NA	1.627***	0.004	NA	NA	9.620*	0.066	-0.889*	0.132	0.0339***
	b3 (dry)	NA	NA	-0.514***	0.000	0.911*	0.056	2.711***	0.000	64.120***	0.000	0.747	0.156	0.0216***
interaction	b12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1.215**	0.044	0.0166***
	b13	NA	NA	0.191*	0.068	NA	NA	NA	NA	NA	NA	NA	NA	-0.017***
	b23	NA	NA	NA	NA	NA	NA	NA	NA	11.660**	0.028	0.924*	0.118	-0.0219***
square	b11	NA	NA	NA	NA	-3.16*	0.010	NA	NA	NA	NA	NA	NA	NA
	b22	NA	NA	NA	NA	NA	NA	NA	NA	17.600*	0.129	NA	NA	-0.123***
	b33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
R^2		0.70	-	0.61	-	0.47	-	0.73	-	0.90	-	0.59	-	0.995
Lack-of-fit		-	0.954	-	0.638	-	0.455	-	0.886	-	0.440	-	0.590	-
The model		-	0.000	-	0.000	-	0.003	-	0.000	-	0.000	-	0.000	-

Note: The explanatory variables were coef - coded coefficients, X_1 : harvest (d), X_2 : inoculum volume (mL), X_3 : membrane type. A stepwise selection of terms was used with $\alpha \leq 0.15$ for a hierarchical model; NA - not applicable, the parameter was removed from the model.

* Significant at $p < 0.15$, **Significant at $p < 0.05$, *** Significant at $p < 0.01$.

The optimization procedure was run against the RSM model taken as a system of equations. The goal was to identify the values of the independent variables (X_1 harvest day, and X_2 inoculum volume) that jointly optimize the fitted dependent variables set as follows: thickness – target to 2 mm; fiber diameter – minimum; swelling half-life – maximum; drug-release half-life – maximum; tensile strength – maximum; Young's modulus – minimum. Thus, the system of equations has with only 1 possible solution, the optimal production conditions in terms of harvest day and inoculum volume. After computing the optimization analysis, our model suggested that the optimum conditions to obtain BC with appropriate properties for biomedical uses might be: X_1 harvest day = 15.70, X_2 inoculum volume = 5 mL, and X_3 membrane type = moist, with a composite desirability = 0.60, when considering harvest intervals of 6 to 18 d, inoculum volumes of 1 to 5 mL, and moist or oven-dried types. Thus, based on the results obtained in the RSM optimization study the following optimum parameters were chosen for further model validation: 16 days of fermentation and 5 mL of inoculum (Table 6) which contains the predicted and the experimental values.

The optimization procedure was run again with X_3 membrane type fixed on “dry” to obtain the optimal solution for BCD, as well. This resulted resulting in X_1 harvest day = 13.64, X_2 inoculum volume = 3.60 mL, with a composite desirability = 0.58. Again, the values were approximated to for $X_1 = 14$ d, $X_2 = 4$ mL for ease of working. The predicted values for the BC properties were obtained after computing the model for the optimum conditions, in each case.

Table 5. The model equations in uncoded values of response variables for each of the 2 types of membranes (dry and moist)

Dry bacterial cellulose:	
$Y_2 = 0.711 + 0.066 \cdot X_1$	(3)
$Y_3 = -4.540 + 2.086 \cdot X_1 + 0.813 \cdot X_2 - 0.088 \cdot X_1^2$	(4)
$Y_4 = 6.891 + 0.164 \cdot X_1$	(5)
$Y_5 = 117.600 - 15.700 \cdot X_2 + 1.991 \cdot X_1 + 4.390 \cdot X_2^2$	(6)
$Y_6 = 55.270 - 0.750 \cdot X_1 - 1.197 \cdot X_2 + 0.101 \cdot X_1 \cdot X_2$	(7)
$D = 0.232 + 0.173 \cdot X_2 + 0.004 \cdot X_1 - 0.031 \cdot X_2^2 + 0.001 \cdot X_2 \cdot X_1$	(8)
Moist bacterial cellulose:	
$Y_2 = 2.504 + 0.002 \cdot X_1$	(9)
$Y_3 = -6.36 + 2.086 \cdot X_1 + 0.813 \cdot X_2 - 0.0879 \cdot X_1^2$	(10)
$Y_4 = 1.468 + 0.164 \cdot X_1$	(11)
$Y_5 = 24.300 + 1.991 \cdot X_1 - 27.400 \cdot X_2 + 4.390 \cdot X_2^2$	(12)
$Y_6 = 56.550 - 0.750 \cdot X_1 - 2.121 \cdot X_2 + 0.101 \cdot X_1 \cdot X_2$	(13)
$D = 0.055 + 0.010 \cdot X_1 + 0.195 \cdot X_2 + 0.001 \cdot X_2 \cdot X_1 - 0.031 \cdot X_2^2$	(14)

Where: X_1 - harvest (d); X_2 - inoculum volume (mL); Y_2 - half-swelling time (h); Y_3 - drug half-release time (h); Y_4 - Tensile strength, (MPa); Y_5 - Young's Modulus (MPa); Y_6 - fiber diameter (μ m); D - desirability function

As it can be observed in Table 6, all validation experimental results fit within the 95% PI intervals, with only one exception, the Young's Modulus of the oven-dried BC. Thus, the model for BCm was validated for all the 5 response variables, while the model for BCd was validated for 4. By setting optimization goals in line with the requirements of a "wound dressing"-use scenario for BC, we obtained and validated the following values for the fermentation parameters: an inoculum volume of 5 mL and a harvest day of 16 for BCm, and an inoculum volume of 4 mL and a harvest day of 14 for BCd.

Table 6. Experimental validation of the mathematical model

$X_1=16$ d, $X_2=5$ mL, and $X_3=$ moist					$X_1=14$ d, $X_2=4$ mL, and $X_3=$ dry			
Resp.	pred.	95% CI	95% PI	exp.	pred.	95% CI	95% PI	exp.
Y1	2.002	(1.751; 2.252)	(1.134; 2.869)	2.110	2.001	(1.835; 2.168)	(1.154; 2.848)	1.960
Y2	2.535	(2.217; 2.852)	(1.474; 3.595)	1.930	1.607	(1.334; 1.880)	(0.559; 2.654)	0.900
Y3	8.800	(6.740; 10.870)	(3.270; 14.34)	3.678	10.500	(8.300; 12.700)	(4.91; 16.08)	7.144
Y4	4.045	(2.992; 5.098)	(0.230; 7.860)	4.770	9.129	(8.161; 10.097)	(5.337; 12.921)	8.126
Y5	28.500	(7.400; 49.600)	(6.300; 83.200)	13.305	145.000	(123.400; 166.600)	(90.100; 200.000)	62.776
Y6	42.120	(39.660; 44.580)	(35.850; 48.390)	43.20	45.705	(44.101; 47.310)	(39.721; 51.689)	45.880

Where: resp. - response variable; pred. - value predicted by the model; exp. - value resulted from the validation experiment; Y1 - thickness (mm); Y2 - half-swelling time (h); Y3 - drug half-release time (h); Y4 - Tensile strength, (MPa); Y5 - Young's Modulus (MPa); Y6 - fiber diameter (μ m)

However, of the 2 types, we would recommend the moist type BC because we managed to validate its production methods for all the 5 response characteristics, but also because its superior mechanical properties, a unique fibrillar structure and water uptake and release properties that makes it suitable for wound dressing (Bodea et al. 2019; Fu et al. 2013; Lin et al. 2013)

Conclusion

The aim of the current study was to optimize the production (fermentation and processing) of BC membranes for properties suitable for biomedical purposes, in general, and specifically for wound dressing material. Two studies were carried out for this purpose: a preliminary study where the medium (100 mL) was inoculated with varying volumes (1; 2; 3; 4; and 5 mL) and incubated statically for different periods (3; 6; 9; 12; and 18 days), using a full factorial experimental design, and an optimization study where a Box-Behnken design was used to assess the effect of 2 independent variables: inoculum volume (X_1 : 1; 3; and 5 mL), fermentation period (X_2 : 6; 12; and 18 d) on each processing (moist, and oven-dried) upon the response variables: thickness, swelling ratio, drug release, fiber diameter, tensile strength, and Young's Modulus. By setting the optimization goals in line with the requirements of a "wound dressing"-use scenario for BC, we obtained and validated the following values for the fermentation parameters: an inoculum volume of 5 and a harvest day of 16 for BCm, and an inoculum volume of 4 mL and a harvest day of 14 for BCd. But, we would recommend the moist type BCm because we manage to validate its production methods for all the 5 response characteristics, but also because its fibrillar network that can incorporate drugs and optimally release them hastening healing. BC has good tension strength and low elongation at break. Additionally, BC maintains proper moisture by either absorbing exudates or releasing and actively participate in tissue regeneration.

Declarations

Acknowledgement

This work was supported by 2 mobility grants of the Romanian Ministry of Research and Innovation, CNCS – UEFISCDI, project number PN-III-P1-1.1-MC-2019-0225 and PN-III-P1-1.1-MC-2019-0213, within PNCDI III. The authors also gratefully acknowledge the support of the Microscopy Service (Servicio de Microscopia) from the Center for Research, Technology and Innovation of the University of Seville (Centro De Investigación, Tecnología E Innovación De La

Universidad De Sevilla), Spain, that granted accesses to the microscopy infrastructure, together with the help and dedication of Cristina Vaquero Aguilar from the Microscopy Service.

References

Addinsoft (2020) XLSTAT statistical and data analysis solution. Long Island, NY, USA

Ahmed A, Saleh A, Soliman N, Ibrahim M, El-Shinnawy N, Abdel-Fattah Y (2019) Biocellulose Production by *Gluconacetobacter hansenii* ATCC 23769: Application of Statistical Experimental Designs and Cellulose Membrane Characterization Egypt J Chem 62:2077-2092 doi:10.21608/ejchem.2019.9975.1662

Almeida I et al. (2014) Bacterial cellulose membranes as drug delivery systems: An in vivo skin compatibility study Eur J Ppharm Biopharm 86:332-336 doi:10.1016/j.ejpb.2013.08.008

Alonso E, Faria M, Mohammadkazemi F, Resnik M, Ferreira A, Cordeiro N (2018) Conductive bacterial cellulose-polyaniline blends: Influence of the matrix and synthesis conditions Carbohydr Polym 183:254-262 doi:10.1016/j.carbpol.2017.12.025

Amin MCIM, Ahmad N, Halib N, Ahmad I (2012) Synthesis and characterization of thermo-and pH-responsive bacterial cellulose/acrylic acid hydrogels for drug delivery Carbohydr Polym 88:465-473 doi:<https://doi.org/10.1016/j.carbpol.2011.12.022>

Ashori A, Sheykhnazari S, Tabarsa T, Shakeri A, Gholipour M (2012) Bacterial cellulose/silica nanocomposites: Preparation and characterization Carbohydrate Polymers 90:413-418 doi:<https://doi.org/10.1016/j.carbpol.2012.05.060>

Badshah M, Ullah H, Khan SA, Park JK, Khan T (2017) Preparation, characterization and in-vitro evaluation of bacterial cellulose matrices for oral drug delivery Cellulose 24:5041-5052 doi:10.1007/s10570-017-1474-8

Bae S, Shoda M (2005) Statistical optimization of culture conditions for bacterial cellulose production using Box-Behnken design Biotechnol Bioeng 90:20-28 doi:10.1002/bit.20325

Barud HS, Souza JL, Santos DB, Crespi MS, Ribeiro CA, Messaddeq Y, Ribeiro SJ (2011) Bacterial cellulose/poly (3-hydroxybutyrate) composite membranes Carbohydr Polym 83:1279-1284 doi:<https://doi.org/10.1016/j.carbpol.2010.09.049>

Basak AK, Aswathy Mol R, Benzegar A, Agunde R, Dinakar S (2015) Absorption of anthocyanin dye and its first order kinetics on bacterial cellulose produced by fermentation of black tea J Biochem Biotechnol 1:28-32 doi:doi:10.6084/M9.FIGSHARE.1488626.V1

Bilgi E, Bayir E, Sendemir-Urkmek A, Hames EE (2016) Optimization of bacterial cellulose production by *Gluconacetobacter xylinus* using carob and haricot bean Int J Biol Macromol 90:2-10 doi:10.1016/j.ijbiomac.2016.02.052

Blanco Parte FG, Santoso SP, Chou CC, Verma V, Wang HT, Ismadji S, Cheng KC (2020) Current progress on the production, modification, and applications of bacterial cellulose Crit Rev Biotechnol 40:397-414 doi:10.1080/07388551.2020.1713721

Bodea IM, Cătuțescu GM, Stroe TF, Dîrlea SA, Beteg FI (2019) Applications of bacterial-synthesized cellulose in veterinary medicine – a review Acta Vet Brno 88:451-471 doi:10.2754/avb201988040451

Cai Z, Kim J (2009) Bacterial cellulose/poly(ethylene glycol) composite: characterization and first evaluation of biocompatibility Cellulose 17:83-91 doi:10.1007/s10570-009-9362-5

Campano C, Balea A, Blanco A, Negro C (2015) Enhancement of the fermentation process and properties of bacterial cellulose: a review Cellulose 23:57-91 doi:10.1007/s10570-015-0802-0

Castro C et al. (2014) In situ production of nanocomposites of poly (vinyl alcohol) and cellulose nanofibrils from *Gluconacetobacter* bacteria: effect of chemical crosslinking Cellulose 21:1745-1756 doi:<https://doi.org/10.1007/s10570-014-0170-1>

Castro C et al. (2012) Bacterial cellulose produced by a new acid-resistant strain of *Gluconacetobacter* genus Carbohydr Polym 89:1033-1037 doi:10.1016/j.carbpol.2012.03.045

Castro C, Zuluaga R, Putaux J-L, Caro G, Mondragon I, Gañán P (2011) Structural characterization of bacterial cellulose produced by *Gluconacetobacter swingsii* sp. from Colombian agroindustrial wastes Carbohydr Polym 84:96-102 doi:10.1016/j.carbpol.2010.10.072

Cătuțescu GM, Rotar AM, Pop CR, Diaconeasa Z, Bunghez F, Socaciu M-I, Semeniuc CA (2019) Influence of extraction pre-treatments on some phytochemicals and biological activity of Transylvanian cranberries (*Vaccinium vitis-idea* L.) Lwt 102:385-392 doi:10.1016/j.lwt.2018.12.062

Costa AFS et al. (2019) Dyeing of bacterial cellulose films using plant-based natural dyes Int J Biol Macromol 121:580-587 doi:10.1016/j.ijbiomac.2018.10.066

Dahman Y, Jayasuriya KE, Kalis M (2010) Potential of biocellulose nanofibers production from agricultural renewable resources: preliminary study Applied biochemistry and biotechnology 162:1647-1659

- Dammström S, Salmén L, Gatenholm P (2005) The effect of moisture on the dynamical mechanical properties of bacterial cellulose/glucuronoxylan nanocomposites *Polymer* 46:10364-10371 doi:<https://doi.org/10.1016/j.polymer.2005.07.105>
- Dayal MS, Goswami N, Sahai A, Jain V, Mathur G, Mathur A (2013) Effect of media components on cell growth and bacterial cellulose production from *Acetobacter acetii* MTCC 2623 *Carbohydr Polym* 94:12-16 doi:<https://doi.org/10.1016/j.carbpol.2013.01.018>
- Du R et al. (2018) Production, Optimization and Partial Characterization of Bacterial Cellulose from *Gluconacetobacter xylinus* TJU-D2 Waste and Biomass Valorization doi:10.1007/s12649-018-0440-5
- Ferreira SLC et al. (2007) Box-Behnken design: An alternative for the optimization of analytical methods *Anal Chim Acta* 597:179-186 doi:10.1016/j.aca.2007.07.011
- Fu L, Zhang J, Yang G (2013) Present status and applications of bacterial cellulose-based materials for skin tissue repair *Carbohydr Polym* 92:1432-1442 doi:10.1016/j.carbpol.2012.10.071
- Fu L et al. (2012) Skin tissue repair materials from bacterial cellulose by a multilayer fermentation method *J Mater Chem* 22:12349 doi:10.1039/c2jm00134a
- Gao W-H, Chen K-F, Yang R-D, Yang F, Han W-J (2011) Properties of bacterial cellulose and its influence on the physical properties of paper *BioResources* 6:144-153 doi:<http://ncsu.edu/bioresources/>
- Garside P, Wyeth P (2003) Identification of cellulosic fibres by FTIR spectroscopy-thread and single fibre analysis by attenuated total reflectance *Stud Conserv* 48:269-275 doi:<https://doi.org/10.1179/sic.2003.48.4.269>
- Gea S, Reynolds CT, Roohpour N, Wirjosentono B, Soykeabkaew N, Bilotti E, Peijs T (2011) Investigation into the structural, morphological, mechanical and thermal behaviour of bacterial cellulose after a two-step purification process *Bioresour Technol* 102:9105-9110 doi:10.1016/j.biortech.2011.04.077
- Goh W, Rosma A, Kaur B, Fazilah A, Karim A, Bhat R (2012) Microstructure and physical properties of microbial cellulose produced during fermentation of black tea broth (Kombucha). *II Int Food Res J* 19:153-158 doi:[http://www.ifrj.upm.edu.my/19%20\(01\)%...](http://www.ifrj.upm.edu.my/19%20(01)%...)
- Hornung M, Ludwig M, Gerrard AM, Schmauder HP (2006) Optimizing the Production of Bacterial Cellulose in Surface Culture: Evaluation of Substrate Mass Transfer Influences on the Bioreaction (Part 1) *Eng Life Sci* 6:537-545 doi:10.1002/elsc.200620162
- Huang C et al. (2015) Evaluating the possibility of using acetone-butanol-ethanol (ABE) fermentation wastewater for bacterial cellulose production by *Gluconacetobacter xylinus* *Lett Appl Microbiol* 60:491-496 doi:<https://doi.org/10.1111/lam.12396>
- Juncu G, Stoica-Guzun A, Stroescu M, Isopencu G, Jinga SI (2016) Drug release kinetics from carboxymethylcellulose-bacterial cellulose composite films *Int J Pharm* 510:485-492 doi:10.1016/j.ijpharm.2015.11.053
- Kirdponpattara S, Khamkeaw A, Sanchavanakit N, Pavasant P, Phisalaphong M (2015) Structural modification and characterization of bacterial cellulose-alginate composite scaffolds for tissue engineering *Carbohydr Polym* 132:146-155 doi:10.1016/j.carbpol.2015.06.059
- Li Y, Jiang H, Zheng W, Gong N, Chen L, Jiang X, Yang G (2015a) Bacterial cellulose-hyaluronan nanocomposite biomaterials as wound dressings for severe skin injury repair *Journal of Materials Chemistry B* 3:3498-3507 doi:10.1039/c4tb01819b
- Li Y, Jiang H, Zheng W, Gong N, Chen L, Jiang X, Yang G (2015b) Bacterial cellulose-hyaluronan nanocomposite biomaterials as wound dressings for severe skin injury repair *J Mater Chem B* 3:3498-3507 doi:10.1039/c4tb01819b
- Li Y, Maciel D, Tomás H, Rodrigues J, Ma H, Shi X (2011) pH sensitive Laponite/alginate hybrid hydrogels: swelling behaviour and release mechanism *Soft Matter* 7:6231 doi:10.1039/c1sm05345k
- Lim J-W, Shin S-M, Jung S-J, Lee MK, Kang SY (2019) Optimization of antibacterial extract from lacquer tree (*Rhus verniciflua* Stokes) using response surface methodology and its efficacy in controlling edwardsiellosis of olive flounder (*Paralichthys olivaceus*) *Aquaculture* 502:40-47 doi:<https://doi.org/10.1016/j.aquaculture.2018.12.008>
- Lin S-P, Kung H-N, Tsai Y-S, Tseng T-N, Hsu K-D, Cheng K-C (2017) Novel dextran modified bacterial cellulose hydrogel accelerating cutaneous wound healing *Cellulose* 24:4927-4937 doi:10.1007/s10570-017-1448-x
- Lin WC, Lien CC, Yeh HJ, Yu CM, Hsu SH (2013) Bacterial cellulose and bacterial cellulose-chitosan membranes for wound dressing applications *Carbohydr Polym* 94:603-611 doi:10.1016/j.carbpol.2013.01.076
- Machado RTA et al. (2016) *Komagataeibacter rhaeticus* as an alternative bacteria for cellulose production *Carbohydr Polym* 152:841-849 doi:10.1016/j.carbpol.2016.06.049
- Nelson ML, O'Connor RT (1964) Relation of certain infrared bands to cellulose crystallinity and crystal lattice type. Part II. A new infrared ratio for estimation of crystallinity in celluloses I and II *J Appl Polym Sci* 8:1325-1341 doi:<https://doi.org/10.1002/app.1964.070080323>

- Nishi Y, Uryu M, Yamanaka S, Watanabe K, Kitamura N, Iguchi M, Mitsunashi S (1990) The structure and mechanical properties of sheets prepared from bacterial cellulose *J Mater Sci* 25:2997-3001 doi:<https://doi.org/10.1007/BF01139032>
- Otto M (2016) *Chemometrics: statistics and computer application in analytical chemistry*. John Wiley & Sons,
- Pal CBT, Jadeja GC (2019) Microwave-assisted deep eutectic solvent extraction of phenolic antioxidants from onion (*Allium cepa* L.) peel: a Box–Behnken design approach for optimization *J Food Sci Technol* 56:4211-4223 doi:10.1007/s13197-019-03891-7
- Paşcalău V et al. (2012) The alginate/k-carrageenan ratio's influence on the properties of the cross-linked composite films *J Alloys Compd* 536:S418-S423 doi:<https://doi.org/10.1080/14686996.2018.1430981>
- Pourjavaher S, Almasi H, Meshkini S, Pirsā S, Parandi E (2017) Development of a colorimetric pH indicator based on bacterial cellulose nanofibers and red cabbage (*Brassica oleracea*) extract *Carbohydr Polym* 156:193-201 doi:10.1016/j.carbpol.2016.09.027
10. Rebelo A, Archer AJ, Chen X, Liu C, Yang G, Liu Y (2018) Dehydration of bacterial cellulose and the water content effects on its viscoelastic and electrochemical properties *Sci Technol Adv Mater* 19:203-211 doi: <https://doi.org/10.1080/14686996.2018.1430981>
- Rambo CR, Recouvreur DOS, Carminatti CA, Pitlovanciv AK, Antônio RV, Porto LM (2008) Template assisted synthesis of porous nanofibrous cellulose membranes for tissue engineering *Mater Sci Eng C* 28:549-554 doi:10.1016/j.msec.2007.11.011
- Rangaswamy B, Vanitha K, Hungund BS (2015) Microbial cellulose production from bacteria isolated from rotten fruit *Int J Polym Sci* 2015:1-8 doi:<https://doi.org/10.1155/2015/280784>
- Rani MU, Rastogi NK, Appaiah KA (2011a) Statistical optimization of medium composition for bacterial cellulose production by *Gluconacetobacter hansenii* UAC09 using coffee cherry husk extract—an agro-industry waste *J Microbiol Biotechnol* 21:739-745 doi:doi:10.4014/jmb.1012.12026
- Rani MU, Udayasankar K, Appaiah KA (2011b) Properties of bacterial cellulose produced in grape medium by native isolate *Gluconacetobacter* sp *J Appl Polym Sci* 120:2835-2841 doi:<https://doi.org/10.1002/app.33307>
- Reiniati I, Hrymak AN, Margaritis A (2017) Recent developments in the production and applications of bacterial cellulose fibers and nanocrystals *Crit Rev Biotechnol* 37:510-524 doi:10.1080/07388551.2016.1189871
- Rodsamran P, Sothornvit R (2019) Extraction of phenolic compounds from lime peel waste using ultrasonic-assisted and microwave-assisted extractions *Food Biosci* 28:66-73 doi:<https://doi.org/10.1016/j.fbio.2019.01.017>
- Sanchavanakit N, Sangrungraungroj W, Kaomongkolgit R, Banaprasert T, Pavasant P, Phisalaphong M (2006) Growth of human keratinocytes and fibroblasts on bacterial cellulose film *Biotechnol Prog* 22:1194-1199 doi: <https://doi.org/10.1021/bp060035o>
- Santoso SP, Chou C-C, Lin S-P, Soetaredjo FE, Ismadji S, Hsieh C-W, Cheng KC (2020) Enhanced production of bacterial cellulose by *Komactobacter intermedius* using statistical modeling *Cellulose* 27:2497-2509 doi:10.1007/s10570-019-02961-5
- Skvortsova ZN, Gromovych TI, Grachev VS, Traskin VY (2019) Physicochemical Mechanics of Bacterial Cellulose Colloid J+ 81:366-376 doi:10.1134/s1061933x19040161
- Sokolnicki AM, Fisher RJ, Harrah TP, Kaplan DL (2006) Permeability of bacterial cellulose membranes *J Membr Sci* 272:15-27 doi:10.1016/j.memsci.2005.06.065
- Sun D, Yang J, Wang X (2010) Bacterial cellulose/TiO₂ hybrid nanofibers prepared by the surface hydrolysis method with molecular precision *Nanoscale* 2:287-292 doi:10.1039/B9NR00158A
- Tan LL, Ren L, Cao YY, Chen XL, Tang XY (2012) Bacterial Cellulose Synthesis in Kombucha by *Gluconacetobacter* sp and *Saccharomyces* sp *Adv Mat Res* 554-556:1000-1003 doi:10.4028/www.scientific.net/AMR.554-556.1000
- Tercjak A, Gutierrez J, Barud HS, Domenegueti RR, Ribeiro SJ (2015) Nano-and macroscale structural and mechanical properties of in situ synthesized bacterial cellulose/PEO-b-PPO-b-PEO biocomposites *ACS Appl Mater Interfaces* 7:4142-4150 doi:<https://doi.org/10.1021/am508273x>
- Ullah H, Badshah M, Mäkilä E, Salonen J, Shahbazi M-A, Santos HA, Khan T (2017) Fabrication, characterization and evaluation of bacterial cellulose-based capsule shells for oral drug delivery *Cellulose* 24:1445-1454 doi:10.1007/s10570-017-1202-4
- Volova TG, Shumilova AA, Shidlovskiy IP, Nikolaeva ED, Sukovaty AG, Vasiliev AD, Shishatskaya EI (2018) Antibacterial properties of films of cellulose composites with silver nanoparticles and antibiotics *Polym Test* 65:54-68 doi:10.1016/j.polymertesting.2017.10.023
- Wang J, Tavakoli J, Tang Y (2019) Bacterial cellulose production, properties and applications with different culture methods - A review *Carbohydr Polym* 219:63-76 doi:10.1016/j.carbpol.2019.05.008
- Wang S, Jiang F, Xu X, Kuang Y, Fu K, Hitz E, Hu L (2017) Super-Strong, Super-Stiff Macrofibers with Aligned, Long Bacterial Cellulose Nanofibers *Adv Mater* 29 doi:10.1002/adma.201702498

Watanabe K, Tabuchi M, Morinaga Y, Yoshinaga F (1998) Structural features and properties of bacterial cellulose produced in agitated culture *Cellulose* 5:187-200 doi:<https://doi.org/10.1023/A:1009272904582>

Wei B, Yang G, Hong F (2011) Preparation and evaluation of a kind of bacterial cellulose dry films with antibacterial properties *Carbohydr Polym* 84:533-538 doi:10.1016/j.carbpol.2010.12.017

Wu CN, Fuh SC, Lin SP, Lin YY, Chen HY, Liu JM, Cheng KC (2018) TEMPO-Oxidized Bacterial Cellulose Pellicle with Silver Nanoparticles for Wound Dressing *Biomacromolecules* 19:544-554 doi:10.1021/acs.biomac.7b01660

Wu J, Zheng Y, Wen X, Lin Q, Chen X, Wu Z (2014) Silver nanoparticle/bacterial cellulose gel membranes for antibacterial wound dressing: investigation in vitro and in vivo *Biomed Mater* 9:035005 doi:10.1088/1748-6041/9/3/035005

Yassine F, Bassil N, Chokr A, El Samrani A, Serghei A, Boiteux G, El Tahchi M (2016) Two-step formation mechanism of *Acetobacter* cellulosic biofilm: synthesis of sparse and compact cellulose *Cellulose* 23:1087-1100 doi:<https://doi.org/10.1007/s10570-016-0884-3>

Yoshino A, Tabuchi M, Uo M, Tatsumi H, Hideshima K, Kondo S, Sekine J (2013) Applicability of bacterial cellulose as an alternative to paper points in endodontic treatment *Acta Biomater* 9:6116-6122 doi:10.1016/j.actbio.2012.12.022

Yu J et al. (2016) Production of Hollow Bacterial Cellulose Microspheres Using Microfluidics to Form an Injectable Porous Scaffold for Wound Healing *Adv Healthc Mater* 5:2983-2992 doi:10.1002/adhm.201600898

Zeinali Kalkhoran AH, Naghib SM, Vahidi O, Rahmanian M (2018) Synthesis and characterization of graphene-grafted gelatin nanocomposite hydrogels as emerging drug delivery systems *Biomed Phys Eng Express* 4:055017 doi:10.1088/2057-1976/aad745

Zeng M, Laromaine A, Roig A (2014) Bacterial cellulose films: influence of bacterial strain and drying route on film properties *Cellulose* 21:4455-4469 doi:10.1007/s10570-014-0408-y

Zeng X, Small DP, Wan W (2011) Statistical optimization of culture conditions for bacterial cellulose production by *Acetobacter xylinum* BPR 2001 from maple syrup *Carbohydr Polym* 85:506-513 doi:10.1016/j.carbpol.2011.02.034

Zhang CJ, Wang L, Zhao JC, Zhu P (2011) Effect of Drying Methods on Structure and Mechanical Properties of Bacterial Cellulose Films *Adv Mat Res* 239-242:2667-2670 doi:10.4028/www.scientific.net/AMR.239-242.2667

Figures

Figure 1

TEM images of *Gluconacetobacter xylinus* cellulose forming bacteria in pristine bacterial cellulose membranes. a - rod shaped bacteria; b – ellipsoidal bacteria; c – cell structure and wall aspect of *G. xylinus*

Figure 2

The response surface plot of thickness performed on the factorial design with 5 levels

Figure 3

FTIR spectra of bacterial cellulose and filter paper on 650-3600 cm^{-1} domain. PF – filter paper, i – inoculum, d – harvest day, M – moist membrane, D – dry membrane; the number following “i” stands for inoculum volume 1, 3, 5 mL and the number following “d” represents the harvest day 6, 12, 18 days.

Figure 4

Swelling ratio of dry and moist bacterial cellulose (BC) membranes over 24 h. a - moist BC membrane; b – dry BC membrane; i – inoculum, d – harvest day; the number following “i” stands for inoculum volume 1, 3, 5 mL and the number following “d” represents the harvest day 6, 12, 18 days.

Figure 5

Moisture content of dry and moist BC membranes over 24 h. a - moist BC membrane; b – dry BC membrane; i – inoculum, d – harvest day; the number following “i” stands for inoculum volume 1, 3, 5 mL and the number following “d” represents the harvest day 6, 12, 18 days.

Figure 6

Drug release activity of BCd and BCm membranes over 72 h; i – inoculum, d – harvest day, M – moist membrane, D – dry membrane; the number following “i” stands for inoculum volume 1, 3, 5 mL and the number following “d” represents the harvest day 6, 12, 18 days.

Figure 7

Kruskal-Wallis graphic test showing fiber diameter differences among BC dry and moist membranes. i – inoculum, d – harvest day, M – moist membrane, D – dry membrane; the number following “i” stands for inoculum volume 1, 3, 5 mL and the number following “d” represents the harvest day 6, 12, 18 days.

Figure 8

Response Surface Methodology RSM contour plots of Y3: drug half-release time of moist (a) and dried (b) bacterial cellulose (BC); Y5: Young's modulus of moist (c) and dried (d) BC; Y6: fiber diameter of moist (e) and dried (f) BC

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Bodeaetal2020RevisedSupplementarydata.docx](#)