A Novel Approach Using Conventional Methodologies to Scale up BNC Production Using Komagataeibacter medellinensis and Rotten Banana Waste as Alternative

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Keywords: BNC scale-up strategy, BNC production optimization, agro-food waste, rotten banana

Abstract:

Currently, cellulose nanostructures are among the most promising structures, and extensive work in materials and biotechnology industries is aimed at identifying an efficient process of production. Even when production at the laboratory scale is successful, crucial aspects of increased commercial applications for cellulose nanostructures are linked to large-scale production. Large-scale production requires a balance between the cost of the culture medium and product value. Therefore, in this work, for the optimization and scaling up of bacterial nanocellulose, a culture medium consisting of rotten banana unsuitable for human consumption was used for the first time as an inexpensive feedstock. Initially, the bacterial nanocellulose (BNC) culture medium conditions were optimized, and it was established that a glucose concentration of 26.4 g/L and a V/A ratio of 2.2 cm were the optimal conditions for production reaching a BNC yield of 5 g/L, which was 42.4% higher than the best result initially obtained. Finally, the scale-up process was performed, implementing a regime analysis methodology by comparing the characteristic times of the critical mechanisms involved in BNC production, namely, microbial growth, glucose consumption, BNC production, and glucose diffusion into the BNC membrane, as the first approach for this type of BNC production process. The mechanism underlying the BNC production process is glucose diffusion into the BNC membrane (characteristic time, 675.47 h). Thus, the V/A ratio was selected as the scale-up criterion most suitable for producing BNC under static culture conditions, allowing the production of 16 g of BNC after 12 d of fermentation in a plastic bioreactor, which was 3378% higher than that produced in glass vessels. The results obtained in this study may initiate further improvements in BNC commercial production by exploiting different feedstocks.

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Article

A Novel Approach Using Conventional Methodologies to Scale up BNC Production Using *Komagataeibacter medellinensis* and Rotten Banana Waste as Alternative

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Abstract: Currently, cellulose nanostructures are among the most promising structures, and extensive work in materials and biotechnology industries is aimed at identifying an efficient process of production. Even when production at the laboratory scale is successful, crucial aspects of increased commercial applications for cellulose nanostructures are linked to large-scale production. Large-scale production requires a balance between the cost of the culture medium and product value. Therefore, in this work, for the optimization and scaling up of bacterial nanocellulose, a culture medium consisting of rotten banana unsuitable for human consumption was used for the first time as an inexpensive feedstock. Initially, the bacterial nanocellulose (BNC) culture medium conditions were optimized, and it was established that a glucose concentration of 26.4 g/L and a V/A ratio of 2.2 cm were the optimal conditions for production reaching a BNC yield of 5 g/L, which was 42.4% higher than the best result initially obtained. Finally, the scale-up process was performed, implementing a regime analysis methodology by comparing the characteristic times of the critical mechanisms involved in BNC production, namely, microbial growth, glucose consumption, BNC production, and glucose diffusion into the BNC membrane, as the first approach for this type of BNC production process. The mechanism underlying the BNC production process is glucose diffusion into the BNC membrane (characteristic time, 675.47 h). Thus, the V/A ratio was selected as the scale-up criterion most suitable for producing BNC under static culture conditions, allowing the production of 16 g of BNC after 12 d of fermentation in a plastic bioreactor, which was 3378% higher than that produced in glass vessels. The results obtained in this study may initiate further improvements in BNC commercial production by exploiting different feedstocks.

Keywords: rotten banana; agro-food waste; BNC production optimization; BNC scale-up strategy

1. Introduction

Bacterial nanocellulose (BNC) is a polymer of glucose units linked by β -1,4 glycosidic linkages, with specific properties such as high crystallinity, nanometric dimensions, and biological compatibility and lignin- and hemicellulose-free [1], making BNC a highly attractive material in industrial sectors such as food [2], paper [3], textile [4], medical devices [5] or composite materials [6]. Despite the



different application fields of BNC, commercial exploitation has been developed in Southeast Asian to produce the dessert nata de coco (bacterial cellulose produced from coconut water as culture medium), which is an good export of these countries [7,8]. However, nata de coco production is developed on a small scale in a traditional way, which leads to low profitability.

Due to the above, different approaches have been sought to make NBC production more technical and increasing the scale focuses on the design of new devices [9,10], the implementation of conventional reactors such as airlift reactors [11], spherical airlift [12] or stirred-tank reactor [13], and in static mode [14,15].

A different approach is to reduce the high cost of the feedstocks used in commercial culture media, such as Hestrin–Schramm (HS) medium [16]. In the last 20 years, the use of agro-industrial waste to produce BNC has been explored, from by-products in the corn industry such as corn steep liquor (CSL) [17] to banana fruit residues [18,19], to improve the production of BNC and reduce production costs [20], allowing the migration from culture broths commonly used to produce BNC, such as the Hestrin–Schramm (HS) medium [21]. In addition to the economic benefits of using agro-industrial waste, with up to 30% of the process cost saved [22,23], the environment and microorganism nutritional requirements are also positively impacted by the employment of this type of feedstock [24]. According to FAO, approximately one-third of all food produced globally is lost or wasted, and this risk is more palpable in developing countries [25]. Banana is produced in 135 countries with an annual production of 145 million tons [26], thus, approximately 48 million tons of this crop may be wasted or lost worldwide. Bananas have valuable nutritional components, including vitamins A, B1, B2 and C, minerals such as Na, Fe, Ca, and P, and a high K content [27–31] which can persist after sterilization process [32], supporting the major nutritional demand of the microbial cultures used to produce BNC

Static culture fermentation is favorable in BNC production because the transformation of productive strains to nonproducing strains is avoided, an action that may be more notable in *Komagataeibacter medellinensis* NBRC 3288 due to an adaptive mutation related to BNC production, as demonstrated by Marsutani et al. (2015) [33,34]. However, conventional scale-up strategies are focused on stirring conditions, which do not apply to static culture processes [35,36]. Therefore, the implementation of scale-up methods such as the rule of thumb, dimensional analysis, trial/error, or regime analysis is novel in processes such as BNC production.

Thus, this study aimed to determine the optimum culture conditions to produce BNC using rotten bananas as a low cost, eco-friendly novel and promising feedstock that supplies all macro- and micronutrients, as well as scale-up BNC production in static cultures economically and technically, providing a basis for further investigation into the commercial production of BNC.

2. Materials and Methods

2.1. Microorganisms and Seed Culture

K. medellinensis NBRC 3288 was obtained from homemade vinegar in Medellín, Colombia, by bioprospecting performed by Castro et al. (2013) [37]. In this study, we used the strains stored in vials in HS medium [21] at pH 3.5 and 20% glycerol (v/v) at 80 °C. The seed culture medium contained (w/v) 0.54% glucose, 0.5% peptone, 0.5% yeast extract, 0.059% KH₂PO₄, 0.025% MgSO₄, 0.267% NaH₂PO₄, and a pH of 5.18 [adjusted with citric acid, 0.2%], and 3% (v/v) of the enzyme Celluclast 1.5 L, added after sterilization. For inoculum production, one vial was added to 100 mL of seed culture medium and agitated in an orbital shaker at 120 rpm for 24, 36, and 48 h.

2.2. Rotten Banana Culture Medium and Production of BNC

Rotten bananas unsuitable for human consumption were acquired at the municipal marketplace of Medellín, Colombia. The culture medium with rotten bananas as the feedstock was obtained by blending by 1:1 ratio of rotten bananas and water. The glucose concentration was adjusted by dilution

of the culture medium, and the pH was adjusted in 3.6 with citric acid, followed by sterilization for 15 min at 121 °C and 15 psi. Subsequently, *K. medellinensis* was transferred to the rotten banana sterilized medium for an inoculum of 10% (v/v) for 7 d under static conditions at room temperature in glass vessels with different volume/area ratios. When the fermentation was completed, the BNC films produced were removed from the surface and added to a solution of 5% (*wt./v*) KOH to remove cells and debris. Finally, the films were rinsed many times with distilled water until a neutral pH was reached. Subsequently, the BNC membranes were dried at 50 °C in a convection oven until a constant weight was measured using an analytical balance.

2.3. Design of Experiments

In the design of the experiments (DoE), we evaluated five factors with a significant influence on the BNC yield based on previous findings [33,38,39]—glucose concentration (g/L), inoculum age (h), inoculum concentration (g/L), ethanol concentration (g/L) and volume/area ratio (cm). The measurement method for each of the evaluated factors is described below.

The samples, blank, and standard were prepared using the method described by BioSystems (Barcelona, Spain). The glucose concentration was measured using the BioSystems glucose (glucose oxidase/peroxide) colorimetric kit. The absorbance was measured at 500 nm using the Thermo[®] UV-VIS Evolution 600 spectrophotometer. The glucose concentration was calculated using Equation (1) as follows:

$$C_{Sample} = C_{Std} \frac{A_{Sample}}{A_{std}} \tag{1}$$

where A_{sample} is the absorbance of the study sample, A_{std} is the absorbance of the kit's standard, C_{std} is the concentration of the kit's standard and equivalent to 1 g/L, and C_{sample} is the concentration of glucose in the considered sample. All the values have a deviation lower than 5%.

Inoculum age was measured according to growth curves obtained previously [40]. To evaluate the significance of this factor, we selected 24 h (exponential phase), 36 h (final exponential phase), and 48 h (begin of stationary phase) as the levels.

Inoculum concentration: The seed culture was centrifuged at 9000 rpm for 20 min at 25–26 °C, and the pellet was collected and resuspended in sterilized saline solution to measure the optical density (OD) using a UV spectrophotometer at 600 nm (OD₆₀₀). Next, using a calibration curve, the concentration of the inoculum was adjusted to 0.1, 0.2 and 0.3 g dry weight/L, and then, the inoculum was transferred to the rotten banana medium at 10% (v/v) for BNC fermentation.

Ethanol concentration: Absolute ethanol (Merck) was added to the rotten banana culture medium at 0% and 0.1% v/v. All the samples were prepared according to the test-combination method described by Boehringer Manheim/R-Biopharm (Roche, Darmstadt, Germany). The ethanol concentration was measured using an ethanol UV-method to determine the ethanol in foodstuffs and other materials using the test-combination method. Ethanol was added after the sterilization process to prevent evaporation.

The volume/area ratio corresponds to the culture medium height in the vessel. Initially, in the screening of the experimental design, this ratio was evaluated in glass vessels with the following surface areas: 22 cm², 30 cm², and 45 cm². The volume of the rotten banana culture medium was 90 cm³. For the optimization stage, the volume/area ratio was varied by modification of the culture medium volume in the glass vessel with a surface area of 45 cm².

2.4. Fractional Factorial Design

Initially, the experimental design was performed using a fractional factorial design $2^{(5-1)}$ plus four center points in duplicate as the screening design to identify factors with a significant influence on the BNC yield. The factor levels are shown in Table 1. The 20 runs of the fractional factorial design $2^{(5-1)}$ plus the center points and results of the BNC yield are shown in Table 2.

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Factor	Coded Name	Levels		
		Low	High	
Glucose concentration (g/L)	X ₁	10.0	30.0	
Ethanol concentration (g/L)	X ₂	0.0	1.0	
Inoculum concentration (g/L)	X ₃	0.14	0.42	
Inoculum age (h)	X_4	24	48	
Volume/area ratio (cm)	X ₅	2.0	4.0	

Table 1. Factor levels evaluated in the screening design.

Table 2.	. Factor	levels eva	aluated in	the screet	ning desigi	n and	results	of the b	acterial	nanoce	llulose ((BNC)
yield at	each lev	vel.										

Run		Code	ed Fact	BNC Vield (g/I)		
Kull	X ₁	X ₂	X ₃	X ₄	X ₅	DIVC Held (g/L)
1	30.0	0.0	0.14	24	2.0	2.34
2	30.0	0.0	0.42	48	2.0	2.29
3	30.0	1.0	0.14	24	4.0	1.89
4	30.0	1.0	0.14	48	2.0	1.75
5	30.0	1.0	0.42	48	4.0	2.03
6	20.0	0.5	0.28	36	3.0	1.94
7	30.0	1.0	0.42	24	2.0	2.88
8	10.0	0.0	0.14	24	4.0	0.71
9	10.0	1.0	0.14	24	2.0	1.35
10	10.0	1.0	0.42	48	2.0	1.04
11	30.0	0.0	0.42	24	4.0	1.81
12	30.0	0.0	0.14	48	4.0	1.92
13	10.0	0.0	0.42	24	2.0	1.12
14	20.0	0.5	0.28	36	3.0	1.92
15	10.0	1.0	0.42	24	4.0	0.82
16	10.0	0.0	0.42	48	4.0	0.76
17	20.0	0.5	0.28	36	3.0	1.88
18	20.0	0.5	0.28	36	3.0	1.81
19	10.0	1.0	0.14	48	4.0	1.01
20	10.0	0.0	0.14	48	2.0	0.48

2.5. Central Composite Design

Once the significant factors were defined (glucose concentration, X_1 , and volume/area ratio, X_5), a central composite experimental design with 16 runs was applied and the results are shown in Table 3.

 Table 3. Factor levels evaluated in the optimization stage and results of the BNC yield.

Run	Coded	Factor	BNC Yield (g/L)			
	X ₁	X ₅				
1	30.0	2.0	3.04			
2	25.0	1.50	3.08			
3	30.0	2.0	3.44			
4	30.0	2.0	3.63			
5	23.0	2.0	3.45			
6	30.0	2.0	3.44			
7	30.0	2.70	3.09			
8	37.0	2.0	2.12			
9	35.0	2.50	2.68			
10	30.0	1.30	2.53			
11	30.0	2.0	3.29			
12	35.0	1.50	1.97			
13	30.0	2.0	3.48			
14	30.0	2.0	3.49			
15	25.0	2.50	3.53			
16	30.0	2.0	3.23			

The second-order model that describes the experimental data is expressed in mathematical form in Equation (2) as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_5 X_5 + \beta_{11} X_1^2 + \beta_{55} X_5^2 + \beta_{15} X_1 X_5$$
(2)

where *Y* is the response (BNC yield), β_0 is the intercept coefficient, β_1 and β_5 are the linear coefficients, β_{11} and β_{55} are the quadratic coefficients, and β_{15} is the interaction coefficient.

The experimental design, statistical analysis, and optimization processes were evaluated using the statistical software package STATGRAPHICS Centurion XVI *(StatPoint Technologies, Inc, The Plains, VA, USA).

2.6. Scale-up of the Bioreactor

To scale-up the fermentation, regimen analysis was performed by comparing the characteristic time, which corresponds to the magnitude of the duration of the BNC production process. The kinetics of different parameters and characteristic times were calculated under optimized culture medium conditions. To determine the glucose consumption, the samples were removed from the culture medium from 0 to 408 h (this was the period during which the glucose consumption was zero), every 48 h. The glucose content was then quantified as described above. The specific consumption rate μ_{Glu} (h⁻¹) was calculated using the fit of the logistic model. The characteristic time of glucose consumption was recorded as the inverse of μ_{Glu} .

Microbial growth: Samples of the BNC membranes were removed from the surface every 48 h until the end of the fermentation process at 408 h. After collecting the BNC membranes, they were washed in sterile saline solution for 3 h under vigorous agitation conditions to remove viable cells in the membrane and complete the cleaning process of the membrane. Next, 1 mL samples from the saline solution were removed to measure the CFU in HS agar medium. The specific growth rate μ_m (h⁻¹) was calculated using the fit of the modified Gompertz's model [41]. The characteristic time of glucose consumption was recorded as the inverse of μ_m .

BNC production: Once the viable cells were removed, the BNC membranes were added to the solution of KOH 5% wt./v to remove biomass and debris, followed by washing until neutral pH was reached. Finally, the BNC membranes were dried in a convection oven until a constant weight was measured in an analytical balance. The specific production rate μ_P (h⁻¹) was calculated using the fit of the logistic model. The characteristic time of glucose consumption was recorded as the inverse of μ_P .

Diffusion of glucose through the BNC membrane: Vertical Franz cells were used to perform the permeation assay of glucose through the BNC membrane. The acceptor chamber was filled with saline solution, and the donor chamber was filled with rotten banana juice. Samples of 1 mL were taken from the acceptor chamber and replaced with the same volume of saline solution. The glucose concentration in the banana juice was adjusted at 20 g/L and replaced every 15 min during the assay to maintain the steady-state condition [42]. Equation (3) [43] was used to calculate the diffusivity coefficient.

$$\frac{M_T}{M_0} = 2 \left(\frac{D_t}{\pi L^2}\right)^2 \tag{3}$$

where M_T is the amount of glucose in the acceptor chamber, M_0 is the amount of glucose in the donor chamber, D is the diffusivity coefficient, and L is the thickness of the BNC membrane.

The scale-up was performed in a polypropylene container with the dimensions of 62.5 cm (long), 29 cm (width) and 18.5 cm (height). This container was selected because it is reusable, inexpensive, easy to access, autoclavable, resistant to impact, translucent allowing the fermentation to be monitored, and lightweight and thus easy to transport.

3. Results and Discussion

3.1. Design of the Experiments and Optimization of the BNC Yield

The BNC yield obtained using *K. medellinensis* NBRC 3288 produced in the rotten banana medium was evaluated by changing the concentration of glucose (X_1), inoculum age (X_2), inoculum concentration (X_3), ethanol concentration (X_4) and volume/area ratio (X_5). The BNC yields obtained with each treatment are shown in Table 2. Figure 1 shows that glucose the concentration and volume/area ratio had a significant effect on the BNC yield because glucose is the building block of cellulose [44]; therefore, as expected, the glucose concentration was the most important factor for BNC yield). However, according to previous reports using *K. medellinensis*, the desirable level of the glucose concentration was 20 g/L [45]. In this study, higher glucose concentration supported a higher BNC production (Figure 1), not only because of the presence of glucose but also because of the other components in banana, such as vitamins (A, B₁, B₂, and C), salts (e.g., potassium and magnesium), and amino acids (e.g., histidine and leucine) [27–31,46].



Figure 1. Glucose diffusion phenomenon through the BNC membrane and the effect of the glucose concentration and V/A ratio on the BNC production yield.

These substances could improve the BNC yield as previously demonstrated for vitamin C [47], certain salts such as $MgSO_4$ or $CaCl_2$ [39], B-complex vitamins [48], and amino acids [49]. Additionally, BNC production occurs on the surface, which is in contact with air, causing increased BNC thickness with time during fermentation. Thus, all the nutrients must be transferred to the zone in contact with the air through diffusion, depending on the concentration gradient [50]. Therefore, the glucose concentration has an impact on BNC yield, as demonstrated previously by Molina-Ramírez et al. (2017).

However, the volume/area ratio was necessary because the yield depends on the volume of the culture medium used (as a relationship of the dry cellulose and volume of medium, g/L). BNC production occurs on the interface of the culture medium and air; therefore, the surface area is a significant variable because oxygen transfer is increased, and the surface is increased [33].

Otherwise, the diffusion of compounds, such as glucose, from the liquid culture medium to the BNC-air interface through the BNC membrane is proportional to the surface area of the vessel due to Fick's Law, as demonstrated previously [51]. This behavior would explain the results of this study, where a higher BNC yield was obtained because of the increasing vessel surface area (reducing V/A ratio) (Figure 1), as demonstrated previously by Ruka et al. (2012). All these mechanisms are shown and explained in Figure 1.

Other factors, such as the inoculum age, inoculum concentration, and ethanol concentration, showed no significant effect on the BNC yield. Therefore, for the next stage of the optimization process, we used a 24-h-old inoculum at the inoculum concentration of 0.42 g/L without the addition of ethanol in the culture media.

Figure 2a shows the response surface methodology indicating the optimum conditions, and Figure 2b displays the contour diagram centered on the optimum conditions for BNC production. The second-order equation that describes BNC production behavior as a function of the glucose concentration and the V/A ratio corresponds to Equation (4).



$$BNC(g/L) = -8.32 + 0.54X_1 + 4.39X_5 - 0.01X_1^2 + 0.02X_1X_5 - 1.15X_5^2$$
(4)

Figure 2. (a) Response surface methodology of the BNC yield as a function of the glucose concentration and V/A ratio. (b) Contour diagram.

Finally, to verify the predicted optimum value results, we used the optimized conditions (glucose concentration at 26.4 g/L and V/A ratio at 2.2 cm) and obtained a BNC yield of 5 ± 0.01 g/L, indicating that BNC produced by *K. medellinensis* NBRC 3288 was increased by 42.4% compared with the best result obtained in the screening stage of this study. The experiment was performed in triplicate.

3.2. Scale-up of BNC Production under Static Conditions

To date, scale-up under static conditions for BNC using rotten bananas as the carbon source has not been reported in the literature. Therefore, a technical criterion was proposed for the first time for successful scale-up. Once the optimal culture conditions to produce BNC from the rotten banana medium were established, the next step was to determine the mechanism underlying the BNC production process through the regime analysis. The word "regime" refers to the dominance of a particular mechanism (process) within the system that generates changes in the conditions of the microorganism, affecting the yield of the product of interest [52]. Regime analysis shows which of the mechanisms determines the performance of the system and how this changes with a change of scale

by comparing the characteristic times of each mechanism involved in the system [52,53]. The kinetic parameters to calculate the characteristics times for each mechanism are presented in Table 4.

Mechanism	Parameter	Characteristic Time (h)						
	Transport Phenomena							
Glucose diffusion	$D_{Glu} = 1.22 \times 10^{-13} \text{ m}^2/\text{s}$	675.47						
Conversion Phenomena								
Microbial growth	$\mu_m = 0.06 \text{ h}^{-1}$	15.62						
BNC production	$\mu_P = 0.02 \text{ h}^{-1}$	44.92						
Glucose consumption	$\mu_{Glu} = 0.22 \text{ h}^{-1}$	4.51						

Table 4. Characteristic time for BNC production under optimized culture conditions.

The diffusion coefficient of glucose through the BNC membrane measured in this study was 1.22×10^{-13} m²/s, which is comparable to that obtained by Hornung et al. (2006), who used HS culture medium [51]. As shown in Table 4, all the mechanisms related to microbial processes were faster than the diffusion of glucose through the BNC membrane. Therefore, the mechanism underlying the BNC production process is glucose diffusion.

This finding was likely due to the thickness of the BNC membrane increasing over time during fermentation, indicating that the carbon source becomes the limiting substrate in the BNC production process [51,54]. The results of the diffusion of glucose through the BNC membranes using different thicknesses are shown in Figure 3.



Figure 3. Comparison of the effect of the BNC membrane thickness on glucose permeation, as measured by the amount of glucose diffused through the BNC membrane over time.

As mentioned above, the diffusion of the quantity of a solute is a function of the area through which this phenomenon occurs. Thus, increasing the surface area of the gas–liquid interface could increase the rate transfer of glucose through BNC, as described by Equation (5) [55].

$$N_{Glu} = AD \frac{dC_{Glu}}{dy} \tag{5}$$

where N_{Glu} is the mass transfer rate of glucose, A is the area across which the mass transfer occurs and dC_{Glu}/dy is the concentration gradient of glucose across the BNC membrane. From Equation (5), it can be supposed that using an infinite area would improve the BNC production yield, but this situation is technically not viable. Therefore, the V/A ratio from the optimization stage was selected as the criterion to perform the scaling-up of the BNC production process for the first time, as shown in Figure 4.



Figure 4. Sketch of the 40-fold scale-up developed in this study.

The V/A ratio can increase the area of the air-liquid interface and the glucose transfer rate without affecting BNC production. Considering the aforementioned factors, the scale-up of BNC production proposed in Figure 4 was performed according to the optimized conditions established previously. Table 5 and Figure 5 shows the results obtained in the BNC production process before and after a scaled process in a large plastic bioreactor. The BNC yield in the plastic bioreactor was slightly lower than that in the vessel (small scale) (Table 5). However, this slight reduction occurs because maintaining the same conditions for both scales was not possible [35]. Furthermore, these results agree with those obtained by Chen et al. (2018), whose scale-up BNC production process led to a 23% reduction in BNC yield [13].

Table 5. Comparison of the BNC yield obtained in the glass vessels and plastic bioreactor. The reduction in BNC yield is notable. However, these results were expected because is not possible to obtain the same results in both scales.

	Draz	Dry Yield (in Weight (g) Total Volume, g/L)	Yield (by Surface Area, g/m ²)	Characterization of Samples *					
	Weight (g)			Nanofibers Diameters (nm)	Porous Size Mean (nm)	Maximun Degradation Temperature Rate (°C)	Crystallinity Index (%)	Modulusof Elasticity (MPa)	
Glass vessel (small scale)	0.46	5	109	110.14	20.69	357.5	82.93	1149.84	
Plastic bioreactor	16	4	89	64.38	16.88	353.3	81.56	569.04	

* This information has been taken from Molina-Ramírez et al. (2020).



Figure 5. A comparative images of BNC membranes obtained in (a) small and (b) large scale in this research.

4. Conclusions

The economic obstacle of producing BNC at low cost has been overcome, thus, industrial-scale production can be considered. In this way, the first approach for the scale-up of BNC production from the rotten banana (not suitable for human consumption) as a cheaper feedstock has been confirmed in this study. By applying response surface methodology (RSM), we established that the glucose concentration and V/A ratio are significant factors involved in the BNC optimization process while rotten banana is used as feedstock. The optimized glucose concentration of 26.4 g/L and V/A ratio of 2.2 cm allowed an increased BNC yield, up to 42.4%, compared with that obtained in the screening stage. Finally, the V/A ratio was chosen as the scale-up technical criterion to maintain the BNC yield in the plastic bioreactor, which was 20% less than that obtained in glass vessels. However, the quantity of the BNC obtained in the scale-up was 16 g, 3378% greater than that obtained in glass vessels under optimized conditions. The results obtained in this study showed that it is possible to scale-up the BNC production under static conditions using a technique that can be replicated by other researchers and with other types of feedstocks.

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References

- Fan, X.; Gao, Y.; He, W.; Hu, H.; Tian, M.; Wang, K.; Pan, S. Production of nano bacterial cellulose from beverage industrial waste of citrus peel and pomace using Komagataeibacter xylinus. *Carbohydr. Polym.* 2016, 151, 1068–1072. [CrossRef] [PubMed]
- 2. Shi, Z.; Zhang, Y.; Phillips, G.O.; Yang, G. Utilization of bacterial cellulose in food. *Food Hydrocoll.* **2014**, 35, 539–545. [CrossRef]

- Santos, S.M.; Carbajo, J.M.; Gómez, N.; Quintana, E.; Ladero, M.; Sánchez, A.; Chinga-Carrasco, G.; Villar, J.C. Use of bacterial cellulose in degraded paper restoration. Part II: Application on real samples. *J. Mater. Sci.* 2015, *51*, 1553–1561. [CrossRef]
- 4. Gayathry, G.; Gopalaswamy, G. Production and characterisation of microbial cellulosic fibre from Acetobacter xylinum. *Indian J. Fibre Text. Res.* **2014**, *39*, 93–96. Available online: http://hdl.handle.net/123456789/27372 (accessed on 15 November 2020).
- Cherian, B.M.; Leão, A.L.; de Souza, S.F.; de Olyveira, G.M.; Costa, L.M.; Brandão, C.V.; Narine, S.S. Bacterial Nanocellulose for Medical Implants. In *Advances in Natural Polymers*; Springer: Berlin/Heidelberg, Germany, 2013; Volume 18, pp. 337–359.
- 6. Shah, N.; Ul-Islam, M.; Khattak, W.A.; Park, J.K. Overview of bacterial cellulose composites: A multipurpose advanced material. *Carbohydr. Polym.* **2013**, *98*, 1585–1598. [CrossRef]
- Phisalaphong, M.; Tran, T.-K.; Taokaew, S.; Budiraharjo, R.; Febriana, G.G.; Nguyen, D.-N.; Chu-Ky, S.; Dourado, F. Nata de coco Industry in Vietnam, Thailand, and Indonesia. In *Bacterial Nanocellulose: From Biotechnology to Bio-Economy*; Elsevier Inc.: Amsterdam, The Netherlands, 2016; pp. 231–236.
- 8. Piadozo, M.E.S. Nata de Coco Industry in the Philippines. In *Bacterial Nanocellulose: From Biotechnology to Bio-Economy;* Elsevier Inc.: Amsterdam, The Netherlands, 2016; pp. 215–229.
- Hornung, M.; Ludwig, M.; Schmauder, H.P. Optimizing the Production of Bacterial Cellulose in Surface Culture: A Novel Aerosol Bioreactor Working on a Fed Batch Principle (Part 3). *Eng. Life Sci.* 2007, 7, 35–41. [CrossRef]
- 10. Kralisch, D.; Hessler, N.; Klemm, D.; Erdmann, R.; Schmidt, W. White biotechnology for cellulose manufacturing—The HoLiR concept. *Biotechnol. Bioeng.* **2009**, *105*. [CrossRef]
- 11. Chao, Y.; Ishida, T.; Sugano, Y.; Shoda, M. Bacterial cellulose production by Acetobacter xylinum in a 50-L internal-loop airlift reactor. *Biotechnol. Bioeng.* **2000**, *68*, 345–358. [CrossRef]
- 12. Song, H.-J.; Li, H.; Seo, J.-H.; Kim, M.-J.; Kim, S.-J. Pilot-scale production of bacterial cellulose by a spherical type bubble column bioreactor using saccharified food wastes. *Korean J. Chem. Eng.* **2009**, *26*, 141–146. [CrossRef]
- Chen, G.; Wu, G.; Alriksson, B.; Chen, L.; Wang, W.; Jönsson, L.J.; Hong, F.F. Scale-up of production of bacterial nanocellulose using submerged cultivation. *J. Chem. Technol. Biotechnol.* 2018, 93, 3418–3427. [CrossRef]
- 14. Kongruang, S. Bacterial cellulose production by Acetobacter xylinum strains from agricultural waste products. *Appl. Biochem. Biotechnol.* **2008**, *148*, 245–256. [CrossRef]
- 15. Mustafa, A.; Babaeipour, V.; Roochi, M.; Kranchezar, S. Enhancement of bacterial cellulose (BC) production by optimizing of inoculation condition: RSM approach. *Iran Polym. Petrochem. Inst.* **2014**, 11.
- Shoda, M.; Sugano, Y. Recent advances in bacterial cellulose production. *Biotechnol. Bioprocess Eng.* 2005, 10, 1–8. [CrossRef]
- 17. Naritomi, T.; Kouda, T.; Yano, H.; Yoshinaga, F. Effect of ethanol on bacterial cellulose production from fructose in continuous culture. *J. Ferment. Bioeng.* **1998**, *85*, 598–603. [CrossRef]
- Molina-Ramírez, C.; Castro, C.; Zuluaga, R.; Gañán, P. Physical Characterization of Bacterial Cellulose Produced by Komagataeibacter medellinensis Using Food Supply Chain Waste and Agricultural By-Products as Alternative Low-Cost Feedstocks. J. Polym. Environ. 2017, 26, 830–837. [CrossRef]
- 19. Molina-Ramírez, C.; Cañas-Gutiérrez, A.; Castro, C.; Zuluaga, R.; Gañán, P. Effect of production process scale-up on the characteristics and properties of bacterial nanocellulose obtained from overripe Banana culture medium. *Carbohydr. Polym.* **2020**, *240*, 116341. [CrossRef]
- 20. Kralisch, D.; Hessler, N. Large-Scale Production of BNC: State and Challenges. In *Bacterial NanoCellulose: A Sophisticated Multifunctional Material*; CRC Press: Boca Raton, FL, USA, 2016; pp. 76–105.
- 21. Hestrin, S.; Schramm, M. Synthesis of cellulose by Acetobacter xylinum. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose*. *Biochem. J.* **1954**, *58*, 345–352. Available online: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1269899/ (accessed on 15 November 2020). [CrossRef]
- 22. Cakar, F.; Özer, I.; Aytekin, A.Ö.; Şahin, F. Improvement production of bacterial cellulose by semi-continuous process in molasses medium. *Carbohydr. Polym.* **2014**, *106*, 7–13. [CrossRef]
- Jozala, A.F.; Pértile, R.A.N.; Dos Santos, C.A.; Santos-Ebinuma, V.D.C.; Seckler, M.M.; Gama, F.M.; Pessoa, A. Bacterial cellulose production by Gluconacetobacter xylinus by employing alternative culture media. *Appl. Microbiol. Biotechnol.* 2015, 99, 1181–1190. [CrossRef]

- 24. Lin, C.S.K.; Pfaltzgraff, L.A.; Herrero-Davila, L.; Mubofu, E.B.; Abderrahim, S.; Clark, J.H.; Koutinas, A.; Kopsahelis, N.; Stamatelatou, K.; Dickson, F.; et al. Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective. *Energy Environ. Sci.* 2013, *6*, 426–464. [CrossRef]
- 25. FAO. *Global Food Losses and Food Waste-Extent, Causes and Prevention;* 2011; Available online: http://www.fao. org/3/mb060e/mb060e00.htm (accessed on 15 November 2020).
- 26. Ploetz, R.C.; Evans, E.A. The Future of Global Banana Production. In *Horticultural Reviews*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2015; Volume 43, pp. 311–352.
- 27. Goswami, B.; Borthakur, A. Chemical and biochemical aspects of developing culinary banana (Musa ABB) 'Kachkal'. *Food Chem.* **1996**, *55*, 169–172. [CrossRef]
- Cano, M.P.; De Ancos, B.; Matallana, M.; Cámara, M.; Reglero, G.; Tabera, J. Differences among Spanish and Latin-American banana cultivars: Morphological, chemical and sensory characteristics. *Food Chem.* 1997, 59, 411–419. [CrossRef]
- 29. Mustaffa, R.; Osman, A.; Yusof, S.; Mohamed, S. Physico-chemical changes in Cavendish banana (Musa cavendishiiL var Montel) at different positions within a bunch during development and maturation. *J. Sci. Food Agric.* **1998**, *78*, 201–207. [CrossRef]
- 30. Emaga, T.H.; Andrianaivo, R.H.; Wathelet, B.; Tchango, J.T.; Paquot, M. Effects of the stage of maturation and varieties on the chemical composition of banana and plantain peels. *Food Chem.* **2007**, *103*, 590–600. [CrossRef]
- 31. Aurore, G.; Parfait, B.; Fahrasmane, L. Bananas, raw materials for making processed food products. *Trends Food Sci. Technol.* **2009**, *20*, 78–91. [CrossRef]
- 32. Davey, K.; Cerf, O. Predicting Concomitant Denaturation of Vitamin as Influenced by Combined Process Temperature and pH in Batch and Continuous Flow Sterilization of Liquids. *Food Bioprod. Process.* **1996**, 74, 200–206. [CrossRef]
- 33. Krystynowicz, A.; Czaja, W.; Wiktorowska-Jezierska, A.; Turkiewicz, M.; Bielecki, S. Factors affecting the yield and properties of bacterial cellulose. *J. Ind. Microbiol. Biotechnol.* **2002**, *29*, 189–195. [CrossRef]
- Matsutani, M.; Ito, K.; Azuma, Y.; Ogino, H.; Shirai, M.; Yakushi, T.; Matsushita, K. Adaptive mutation related to cellulose producibility in Komagataeibacter medellinensis (Gluconacetobacter xylinus) NBRC 3288. *Appl. Microbiol. Biotechnol.* 2015, 99, 7229–7240. [CrossRef]
- 35. Ju, L.-K.; Chase, G. Improved scale-up strategies of bioreactors. *Bioprocess Biosyst. Eng.* **1992**, *8*, 49–53. [CrossRef]
- Villadsen, J.; Nielsen, J.; Lidén, G. Scale-Up of Bioprocesses. In *Bioreaction Engineering Principles*; Springer US: Boston, MA, USA, 2011; pp. 497–546.
- Castro, C.; Cleenwerck, I.; Trček, J.; Zuluaga, R.; De Vos, P.; Caro, G.; Aguirre, R.; Putaux, J.-L.; Gañán, P. Gluconacetobacter medellinensis sp. nov., cellulose- and non-cellulose-producing acetic acid bacteria isolated from vinegar. *Int. J. Syst. Evol. Microbiol.* 2013, 63, 1119–1125. [CrossRef]
- 38. Ruka, D.R.; Simon, G.P.; Dean, K. Altering the growth conditions of Gluconacetobacter xylinus to maximize the yield of bacterial cellulose. *Carbohydr. Polym.* **2012**, *89*, 613–622. [CrossRef] [PubMed]
- 39. Mohite, B.V.; Kamalja, K.K.; Patil, S.V. Statistical optimization of culture conditions for enhanced bacterial cellulose production by Gluconoacetobacter hansenii NCIM 2529. *Cellulose* **2012**, *19*, 1655–1666. [CrossRef]
- 40. Molina-Ramírez, C.; Zuluaga, R.; Castro, C.; Gañán, P. Statistical optimization of culture conditions to improve cell density of Komagataeibacter medellinensis NBRC 3288: The first step towards to optimize bacterial cellulose production. *bioRxiv* **2018**, 381186. [CrossRef]
- Zwietering, M.H.; Jongenburger, I.; Rombouts, F.M.; Riet, K.V. T Modeling of the Bacterial Growth Curve. *Appl. Environ. Microbiol.* 1990, 56, 1875–1881. Available online: http://www.ncbi.nlm.nih.gov/pubmed/ 16348228 (accessed on 15 November 2020). [CrossRef] [PubMed]
- Díez-Sales, O.; Copoví, A.; Casabó, V.; Herraez, M. A modelistic approach showing the importance of the stagnant aqueous layers in in vitro diffusion studies, and in vitro-in vivo correlations. *Int. J. Pharm.* 1991, 77, 1–11. [CrossRef]
- 43. Crooks, J.E. Measurement of diffusion coefficients. *J. Chem. Educ.* **1989**, *66*, 614. Available online: https://pubs.acs.org/sharingguidelines (accessed on 15 November 2020). [CrossRef]

- 44. Cocinero, E.J.; Gamblin, D.P.; Davis, B.G.; Simons, J.P. The Building Blocks of Cellulose: The Intrinsic Conformational Structures of Cellobiose, Its Epimer, Lactose, and Their Singly Hydrated Complexes. *J. Am. Chem. Soc.* **2009**, *131*, 11117–11123. [CrossRef]
- 45. Molina-Ramírez, C.; Castro, M.; Osorio, M.; Torres-Taborda, M.; Gómez, B.; Zuluaga, R.; Gómez, C.; Gañán, P.; Rojas, O.J.; Castro, C. Effect of Different Carbon Sources on Bacterial Nanocellulose Production and Structure Using the Low pH Resistant Strain Komagataeibacter Medellinensis. *Materials*. **2017**, *10*, 639. [CrossRef]
- 46. Ito, H.; Ueno, H.; Kikuzaki, H. Free Amino Acid Compositions for Fruits. J. Nutr. Diet. Pract. 2017, 1, 1–5.
- Keshk, S.M. Vitamin C enhances bacterial cellulose production in Gluconacetobacter xylinus. *Carbohydr. Polym.* 2014, 99, 98–100. [CrossRef]
- 48. Kouda, T.; Yano, H.; Yoshinaga, F. Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. *J. Ferment. Bioeng.* **1997**, *83*, 371–376. [CrossRef]
- 49. Matsuoka, M.; Tsuchida, T.; Matsushita, K.; Adachi, O.; Yoshinaga, F. A Synthetic Medium for Bacterial Cellulose Production byAcetobacter xylinumsubsp.sucrofermentans. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 575–579. [CrossRef]
- 50. Laidler, K.J.; Shuler, K.E. The Kinetics of Membrane Processes. I. The Mechanism and the Kinetic Laws for Diffusion through Membranes. *J. Chem. Phys.* **1949**, *17*, 851. [CrossRef]
- Hornung, M.; Ludwig, M.; Gerrard, A.M.; Schmauder, H.-P. Optimizing the Production of Bacterial Cellulose in Surface Culture: Evaluation of Substrate Mass Transfer Influences on the Bioreaction (Part 1). *Eng. Life Sci.* 2006, *6*, 537–545. [CrossRef]
- 52. Sweere, A.; Luyben, K.; Kossen, N. Regime analysis and scale-down: Tools to investigate the performance of bioreactors. *Enzym. Microb. Technol.* **1987**, *9*, 386–398. [CrossRef]
- 53. Mavituna, F. Strategies for Bioreactor Scale-Up. In *Computer and Information Science Applications in Bioprocess Engineering*; Springer Science and Business Media LLC: Dordrecht, The Netherlands, 1996; pp. 125–142.
- Verschuren, P.G.; Cardona, T.D.; Nout, M.; De Gooijer, K.D.; Heuvel, J.C.V.D. Location and limitation of cellulose production by Acetobacter xylinum established from oxygen profiles. *J. Biosci. Bioeng.* 2000, *89*, 414–419. [CrossRef]
- 55. Doran, P.M. Mass Transfer. In *Bioprocess Engineering Principles*; Elsevier: Amsterdam, The Netherlands, 2013; pp. 379–444.

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