New BIOTECHNOLOGY 49 (2019) 19–27

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt

Response surface statistical optimization of bacterial nanocellulose fermentation in static culture using a low-cost medium

Ana Cristina Rodrigues[a], Ana Isabel Fontão[a,1], Aires Coelho[a,1], Marta Leal[a], Francisco A.G. Soares da Silva[a,1], Yizao Wan[b], Fernando Dourado[a,*], Miguel Gama[a]

[a] CEB- Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal
[b] School of Materials Science and Engineering, East China Jiaotong University, Nanchang, 330013, China

ARTICLE INFO

Keywords:
BNC production optimization
Low-cost substrates
Response surface methodology-central composite design
Culture medium depth
Surface area

ABSTRACT

This work aimed at the optimization of bacterial nanocellulose (BNC) production by static culture, using *Komagataeibacter xylinus* BPR 2001 *(K. xylinus)*. Response surface methodology - central composite design was used to evaluate the effect of inexpensive and widely available nutrient sources, namely molasses, ethanol, corn steep liquor (CSL) and ammonium sulphate, on BNC production yield. The optimized parameters for maximum BNC production were % (m/v): molasses 5.38, CSL 1.91, ammonium sulphate 0.63, disodium phosphate 0.270, citric acid 0.115 and ethanol 1.38% (v/v). The experimental and predicted maximum BNC production yields were 7.5 ± 0.54 g/L and 6.64 ± 0.079 g/L, respectively and the experimental and predicted maximum BNC productivity were 0.829 ± 0.046 g/L/day and 0.734 ± 0.079 g/L/day, after 9 days of static culture fermentation, at 30 °C. The effect of surface area and culture medium depth on production yield and productivity were also studied. BNC dry mass production increased linearly with surface area, medium depth and fermentation time. So long as nutrients were still available in the culture media, BNC mass productivity was constant. The results show that a high BNC production yield can be obtained by static culture of *K. xylinus* BPR 2001 using a low-cost medium. These are promising conditions for the static industrial scale BNC production, since as compared to agitated bioreactors, higher productivities may be reached, while avoiding high capital and operating costs.

Introduction

Bacterial nanocellulose (BNC) is an exopolysaccharide produced by *Komagataeibacter xylinus* (formerly *Gluconacetobacter xylinus*), a Gram negative and strictly aerobic bacterium of the *Acetobacteraceae* family [1–6]. BNC shows several unique physicochemical and mechanical properties, including high purity, high crystallinity, high degree of polymerization [7], an ultrafine fiber network, high water holding and absorbing abilities [8], high tensile strength in the wet state [9], and the possibility to be shaped into 3D structures during synthesis. It is biocompatible and biofunctional [10]. Due to these properties, the biopolymer has been studied in several applications, including tissue regeneration, drug delivery systems, vascular grafts, in vitro and in vivo scaffolds for tissue engineering, electronic paper displays and in food applications [11–17]. These properties and applications have generated a growing interest in the development of new strategies aimed at large-scale BNC production. Several fermentation technologies have been attempted, such as agitated, air-lift, membrane and horizontal bioreactors, using different fermentation media and overproducing mutant strains. Stirred tank reactors can prevent the heterogeneity of the culture broth, at the expense of a high energy cost for generation of mechanical power. Airlift reactors typically require only one sixth of the energy power used in stirred tank reactors. Nonetheless, the agitation power of an airlift reactor is limited, resulting in low fluidity of the culture broth, especially at high cellulose concentrations. In addition, both agitation and aeration systems have been reported to result in the development of cellulose-negative mutants (non-cellulose producers, Cel$^-$) [18–20]. In the case of membrane bioreactors, the major drawbacks include high operating costs and difficulty in collecting the cellulose from the reactors following fermentation [9,18–23].

Abbreviations: BNC, bacterial nanocellulose; *K. xylinus*, *Komagataeibacter xylinus*; CSL, corn steep liquor; RSM, Response Surface Methodology; CCD, Central Composite Design; HS medium, Hestrin-Schramm culture medium; S, surface fermentation area; L, culture medium depth; V, culture medium volume

* Corresponding author.
E-mail address: fdourado@deb.uminho.pt (F. Dourado).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.nbt.2018.12.002
Received 5 June 2018; Received in revised form 5 December 2018; Accepted 5 December 2018
Available online 06 December 2018
1871-6784/

"Traditional" static cultivation methods for BNC production, mostly used in Asian countries, are difficult to implement on a large scale. Although the yield is relatively high, the long fermentation times required, the need for large areas and intensive manpower and high labour costs have deterred such processes from implementation in large scale, modern facilities. Alongside the fermentation method (static versus agitated/aerated), which impacts on the capital investment and operating costs), the economic feasibility of BNC production is directly dependent on product yield. The production parameters in static culture include the composition of the culture media, fermentation temperature, pH and time, inoculum ratio [18,24] and surface area to volume ratio (air-liquid interface) of the culture medium (S/V) [20,25]. The greater the medium surface area, the higher the production of BNC, given the aerobic character of the bacterium. Several reports have analysed the optimal surface area/volume ratio for BNC production in static culture [20,25,26], but the results obtained cannot be easily compared, due to differences in fermentation times, culture media composition and strains used. As with many fermentation processes, the cost and availability of the substrates play a determining role in the economic feasibility of the process. Thus, it is important to explore the use of widely available low-cost substrates, especially agro-industrial by-products, to improve BNC yield. While several reports have addressed the use of different culture media to optimize BNC production using *K. xylinus* BPR 2001 under agitated condition, less attention has been paid to the use of static culture specifically for *K. xylinus* BPR 2001. Those studies relied on the use of fructose and corn steep liquor (CSL) as the carbon and nitrogen sources, combined with a large number (sometimes more than 20) of other nutrients, such as different vitamins, amino acids and salts. Such complex culture media are impractical for the large-scale implementation of a BNC production process (Table 1).

The cost of the nutrients, media composition, available surface area, fermentation depth and time, should all be considered for economic BNC production in static culture. Here, we report optimization of BNC production by *K. xylinus* BPR 2001 under static culture conditions, using a simple culture medium composition. Optimization was performed using response surface methodology (RSM) - central composite design (CCD). The effect of four nutrients - molasses and ethanol as the carbon sources, CSL as the nitrogen and protein source and ammonium sulphate - on the BNC production yield (g/L) (as the response variable) was assessed. In addition, the effect of the surface area and culture medium depth on the BNC production yield and productivity, were evaluated.

Materials and methods

Bacterial strain

K. xylinus subsp *sucrofermentants* BPR 2001 (ATCC 700178), from the American Type Culture Collection, was used for the production of BNC under static conditions. The strain was maintained in Hestrin-Schramm culture medium (HS medium) [46], in solid state with 2% (m/v) agar (Acros Organics).

Inoculum preparation and static culture fermentation

BPR 2001 cells were grown in 1 L conical flasks, containing 100 mL HS medium, comprising (in % m/v) glucose 2.0 (Fisher Chemical), peptone 0.5 (OXOID), yeast extract 0.5 (OXOID), disodium phosphate (Na_2HPO_4) 0.27 (Panreac) and citric acid 0.115 (Panreac). The initial pH was set to 5.5 using 18% (v/v) HCL (Fisher-Chemical). The medium was autoclaved at 121 °C, 1 bar for 20 min before use. The culture was incubated for 2 d at 30 °C under static conditions. Thereafter, the cellulose pellicle formed was vigorously shaken in order to remove active

Table 1
Summary of the data available on the BNC production yield with *K. xylinus* BPR2001.

Carbon Source	Nitrogen Source	Type of culture	Additives/ [a]BNC production yield
Fructose	**CSL**	Agitated Jar fermenter (with oxygen suplementation, batch and fed-batch conditions)	• **BNC yield** 7.7 g/L [27]; • [b]Complex medium • endo-p-l,4-glucanase from *Bacillus suhtilis* • **BNC yield** 4.5 g/L [28]; • Agar • **BNC yield** 12.8 g/L [29]; • Complex medium • **BNC yield** 7.5 g/L [30]; • Complex medium • Agar • **BNC yield** 14.3 g/L [31]; • KH_2PO_4 • $(NH_4)_2SO_4$ • $MgSO_4{\cdot}7H_2O$ • **BNC yield** 1.13 g/L [32].
		Agitated Shaken flasks	• Complex medium and Polyacrylamide-co-acrylic acid • **BNC yield** 6.5 g/L [33]; • Complex medium • Carboxymethyl celulose • Microcrystalline celulose • Agar and Sodium alginate • **BNC yield** 8.2 g/L [34].
		Agitated air-lift reactor	• Complex medium • Agar • Xanthan • **BNC yield** 8.7 g/L [35]; • Complex medium • **BNC yield** 3.8 g/L and 10.4 g/L [36,37].
		Agitated Plastic composite support rotating disk bioreactor (PCS-RDB),	• Complex medium • Carboxymethyl celulose • **BNC yield** 13 g/L [38]; • Complex medium • Microcrystalline cellulose (Avicel) • Carboxymethylcellulose (CMC) • Agar • Sodium alginate • **BNC yield** 0.64 g/slice [39].
Treated Molasses		Agitated jar fermenter	• Complex medium • **BNC yield** 14.3 g/L and 12.8 g/L [30,40].
Wheat straw hydrolysate		Agitated Flask shaken	• Complex medium • **BNC yield** 10.6 g/L [41].
Wheat straw hydrolysate **Corn fibers** **Distillller´s dried grains with Solubles**		Agitated Flask shaken	• Complex medium • **BNC yield** 5.2 g/L [42].
Glucose or dextrose		Flask shaken	• 1-Methylcyclopropene • magnesium sulphate • ammonium sulphate • **BNC yield** 1.2 g/L [43].
Maple syrup	Yeast extract	Flask shaken	• Ethanol • Acetic acid • $MgSO_4{\cdot}7H_2O$ • Agar • **BNC yield** 3.2 g/L [44].
Carob	Haricot bean	Static	• **BNC yield** 6.5 g/L [45].

[a]BNC production yield is represented as g dry BNC mass/Litre of culture media.
[b]By complex medium is meant a combination of culture medium to which different vitamins, amino acids and salts were added. Sometimes the culture medium contains about 20 or more components.

cells entrapped within the cellulose matrix; 4 mL (10% (v/v) of the final volume) of this inoculum was transferred to 100 mL conical flasks, containing a final volume of 40 mL of different combinations of culture media, prepared using molasses (a gift from RAR Refinarias de Açúcar Reunidas, S.A.; Portugal), CSL (a gift from COPAM Companhia Portuguesa de Amidos, S.A.; Portugal), ammonium sulphate (Panreac) and ethanol (Fisher-Chemical), as described below. The inoculated media were incubated for 9 d, at 30 °C under static conditions. After cultivation, the BNC membranes were collected, purified and the production yield (in g/L) was determined as described below.

Optimization of BNC production using response surface methodology (RSM) - central composite design

In this study, the optimization process of BNC production firstly entailed identifying the preferred nutrients (carbon and nitrogen sources) for BNC production based on the literature (Table 1) and varying one factor at a time while keeping the others constant (data not shown). Based on the collected information, preliminary fermentation assays were performed to evaluate the effect of the selected nutrients (and their concentrations), on BNC yield. Data collected from these experiments allowed better determination of the boundaries for each variable to be tested (levels of factors). Molasses and CSL are the most economical carbon and nitrogen sources commonly used in industrial fermentations [40,47] (Table 1). CSL is a nutrient-rich by-product supply of amino acids, vitamins and minerals, and has been reported to have buffering capacity [48]. According to the literature review, ethanol and ammonium sulphate have been observed to increase BNC production yield [20,43,49–51]. Also, certain *Acetobacteraceae* strains are known to be capable of using ethanol as an additional carbon source [18,21,49–51]. Supplementing a culture medium with ethanol also allowed repression of the spontaneous mutations of BNC producing cells (Cel^+) to non-producing cells (Cel^-) and increased cells ATP production [20,49–52]. As such, these compounds were included in our media formulations.

The optimization process then focused on evaluating the effect of four independent variables: molasses (A) and ethanol (D) as carbon sources, nitrogen from CSL (B) and ammonium sulphate (C), on the yield of BNC, using response surface methodology based on central composite design (Tables 2 and 3). The software Design Expert 7.1.5 (Stat-Ease, Inc., USA, Windows operating system) was used to determine the experimental design matrix and its statistical experimental design analysis. All the assays/formulations were performed in triplicate, except the central point of the factorial design, were 5 replicates were performed, resulting in a total of 77 experiments and 25 different culture media formulations (Table 3). All combinations of the fermentation medium included 0.27% (m/v) Na_2HPO_4 and 0.115% (m/v) citric acid. The initial pH used was set to 5.5 in all media. HS medium was used as control.

Three-dimensional curves of the response surfaces were generated using Design Expert 7.1.5 (Stat-Ease, Inc., USA, Windows operating system) to visualize individual effects and interaction between significant parameters. All experiments were performed independently, following the sequential order shown in Table 3. Each run was performed in triplicate and an average value of the responses was used for the presentation of the results. The model was evaluated using the Fisher´s statistical test for analysis or variance (ANOVA).

Table 3
Central Composite design matrix for the four variables. Coded values and real values, where coded values given in parentheses.

# Run	A:Molasses % m (total of sugar/v)	B:CSL % m (total of protein/v)	C:Ammonium Sulphate % (m/v)	D:Ethanol % (v/v)
1 |2 |15	(-2) 2.00	(0) 1.33	(0) 1.25	(0) 1.75
4 |55 |72	(-1) 3.13	(+1) 1.91	(+1) 1.88	(-1) 1.38
5 |20 |49	(-1) 3.13	(-1) 0.74	(+1) 1.88	(-1) 1.38
8 |59 |74	(-1) 3.13	(-1) 0.74	(+1) 1.88	(+1) 2.13
14 |32 |68	(-1) 3.13	(+1) 1.91	(-1) 0.63	(-1) 1.38
39 |56 |58	(-1) 3.13	(+1) 1.91	(+1) 1.88	(+1) 2.13
9 |29 |52	(-1) 3.13	(-1) 0.74	(-1) 0.63	(-1) 1.38
33 |60 |66	(-1) 3.13	(-1) 0.74	(-1) 0.63	(+1) 2.13
23 |43 |71	(-1) 3.13	(+1) 1.91	(-1) 0.63	(+1) 2.13
7 |24 |64	(0) 4.25	(+2) 2.50	(0) 1.25	(0) 1.75
12 |16 |40	(0) 4.25	(0) 1.33	(-2) 0.00	(0) 1.75
21 |26 |28 |48 |65	(0) 4.25	(0) 1.33	(0) 1.25	(0) 1.75
27 |34 |62	(0) 4.25	(0) 1.33	(0) 1.25	(+2) 2.50
31 |50 |73	(0) 4.25	(-2) 0.15	(0) 1.25	(0) 1.75
35 |45 |61	(0) 4.25	(0) 1.33	(+2) 2.50	(0) 1.75
51 |54 |70	(0) 4.25	(0) 1.33	(0) 1.25	(-2) 1.00
25 |75 |76	(+1) 5.38	(+1) 1.91	(+1) 1.88	(-1) 1.38
22 |44 |77	(+1) 5.38	(-1) 0.74	(-1) 0.63	(-1) 1.38
10 |63 |69	(+1) 5.38	(+1) 1.91	(-1) 0.63	(+1) 2.13
6 |30 |42	(+1) 5.38	(-1) 0.74	(-1) 0.63	(+1) 2.13
41 |46 |57	(+1) 5.38	(+1) 1.91	(-1) 0.63	(-1) 1.38
11 |36 |67	(+1) 5.38	(+1) 1.91	(+1) 1.88	(+1) 2.13
19 |37 |53	(+1) 5.38	(-1) 0.74	(+1) 1.88	(-1) 1.38
3 |13 |47	(+1) 5.38	(-1) 0.74	(+1) 1.88	(+1) 2.13
17 |18 |38	(+2) 6.50	(0) 1.33	(0) 1.25	(0) 1.75

Effect of surface area at a constant S/V ratio on BNC production yield

The effect of surface fermentation area (S) on BNC yield was evaluated. Containers having variable fermentation areas and a fixed fermentation broth depth (of 2.5 cm) were used, resulting in a fixed S/V ratio of 0.4 cm^{-1}. Fermentation broth containing molasses 4% (m/v), CSL 0.7% (m/v) (protein basis), ethanol 1.5% (v/v), ammonium sulphate 0.5% (m/v), Na_2HPO_4 0.27% (m/v) and citric acid 0.115% (m/v), initial pH 5.5, was sterilized. The medium was then inoculated and transferred to the containers with the different surface areas. These were incubated for 15 d under static conditions at 30 °C. BNC was then collected, purified and the production yield (in g/L) determined as described below.

Effect of surface area/culture medium depth ratio (S/L) on BNC production yield

The effect of the culture medium depth (L) on the BNC yield was evaluated. Containers having the same fermentation area (S, 336 cm^2), were filled with inoculated fermentation broth (as described above) at a depth (L) of 1 cm (320 mL), 2.5 cm (620 mL) and 4 cm (1000 mL), yielding S/L ratios of 336, 134.4 and 84 cm and S/V ratios of 1.05, 0.54

Table 2
Levels of factors chosen for the experimental central composite design.

Sources	Variable	Symbol	Lower Limit (-2)	Low Coded (-1)	Center Coded (0)	High Coded (+1)	Higher Limit (+2)	Units
Carbon	Molasses (total sugars)	(A)	2.00	3.13	4.25	5.38	6.50	% (m/v)
	Ethanol	(D)	1.00	1.38	1.75	2.13	2.50	% (v/v)
Nitrogen	CSL (protein basis)	(B)	0.15	0.74	1.33	1.91	2.50	% (m/v)
	Ammonium Sulphate total	(C)	0.00	0.63	1.25	1.88	2.50	% (m/v)

and 0.34 cm^{-1}, respectively. All were incubated for 9, 15 and 21 d under static conditions at 30 °C. BNC was purified and the production yield (g/L) was determined as described below.

BNC purification and BNC yield determination

After cultivation, the BNC membranes obtained were washed with distilled water at room temperature (RT) to remove culture medium residues, then washed with 0.1 M NaOH (Fisher-Chemical) at RT; this solution was changed twice daily until the membranes turned completely white by visual inspection. The bleached membranes were then washed with distilled water at RT until the pH became that of the distilled water. The purified BNC was oven dried to constant mass at 50 °C and weighed to determine production yield (expressed in g of dry BNC/L of culture media).

Analytical methods- Total sugars and protein quantification

Analysis of total molasses sugars was by HPLC, using a Metacarb 87 H column (300, 7.8 mm, Varian, USA), PU-2080 Plus pump (JASCO), DG-2080-53 degasser (JASCO), AS2057-Plus automatic sample injector (JASCO) and a 2031 Plus RI detector (JASCO) under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.5 mL/min, and column temperature 35 °C (Oven Elder CH-150). The injected volume was 20 µL. The concentrations of sucrose, glucose and fructose were determined based on calibration curves obtained using pure compounds. The composition of molasses (g/L) determined was sucrose 687.7 ± 1.23, glucose 20.6 ± 6.22 and fructose 12.8 ± 2.05.

Total protein analysis of CSL was performed by BCA Protein assays kit (Pierce® BCA 23227 Protein Assay Kit, Thermo Scientific). The total protein CSL composition was 167.5 ± 8.6 g/L.

Statistical analysis

The statistical analyses One-way and Two-way ANOVA were performed using GraphPad Prism version 5 for Windows, GraphPad Software, San Diego, California, USA.

Results and discussion

Response surface methodology – central composite design

A statistically designed study was conducted to investigate the individual and interactive effect of four medium components on BNC yield. The experimental results from the 77 experiments (Table 3) are presented in Fig. 1. The first set of optimal statistical conditions, maximizing BNC production yield by *K. xylinus* BPR 2001 under static culture were obtained with experiments 41, 46 and 57, which corresponded to the same medium composition, i.e. molasses 5.38% (m/v), CSL 1.91% (m/v) (protein basis), ammonium sulphate 0.63% (m/v), ethanol 1.38% (v/v) (Table 3). Under these conditions, 87% of the initial sugars were consumed by the bacteria after 9 d static culture fermentation, as determined by total sugars assay (results not shown). The average BNC production yield and productivity were 7.5 ± 0.54 g/L and 0.829 ± 0.046 g/L/day, respectively (Fig. 1). Independent assays were performed (triplicates) under the optimal conditions and the results for BNC production yield were confirmed ($p > 0.05$). This average yield value, as obtained with a low-cost formulation represents a 6.3 fold increase in BNC production yield compared with the HS medium (Fig. 1, HS control). Interestingly, the experiment trials 51, 54 and 70, corresponding to a medium composition of molasses 4.25% (m/v), CSL 1.33% (m/v) (protein basis), ammonium sulphate 1.25% (m/v) and ethanol 1.0% (v/v), resulted in a similar BNC production yield ($p > 0.05$). These trials generated an average BNC production yield of 7.0 ± 0.25 g/L. While achieving a (statistically) similar yield, in this second set of experiments, with the exception of ammonium sulphate, all other nutrients were used in smaller amounts. This had a positive impact on the cost of the culture media. It should be noted that much higher BNC productivities have been reported in the literature (Table 1), under agitated conditions and using complex medium. However, for industrial production, it is necessary to consider capital investment and operating costs. Scaling up of BNC fermentation implies first the use of increasingly larger seed vessels for inoculum propagation. Under agitated conditions, multiple agitated fermenters (stirred tank or airlift fermenters) also have to be used. Together, this equipment represents a significant capital investment. In addition, high operating costs are involved, associated mainly with the fermenters' operation and cleaning processes. In contrast, the capital investment and operating cost of a cleanroom, for static culture, should be lower [23,53].

RSM is a four factorial design (Table 2) where 3D contour plots or surface curves (Supplementary material, Figure S1 and Fig. 2) can be generated by linear effects, quadratic effects and two-way interactions between the factors. From these profiles, a semi-empiric model (Eq. 1) can be derived that best fits the experimental data. This allows calculation of the optimal responses of the system, in this case the maximum BNC yield. The parameters and results from the CCD experiments are presented in Tables 3 and 4, Fig. 1 and supplementary material Figure S1. The statistical significance of the quadratic model was tested through *F*- and *p*- values (Table 4). Results from ANOVA indicated that

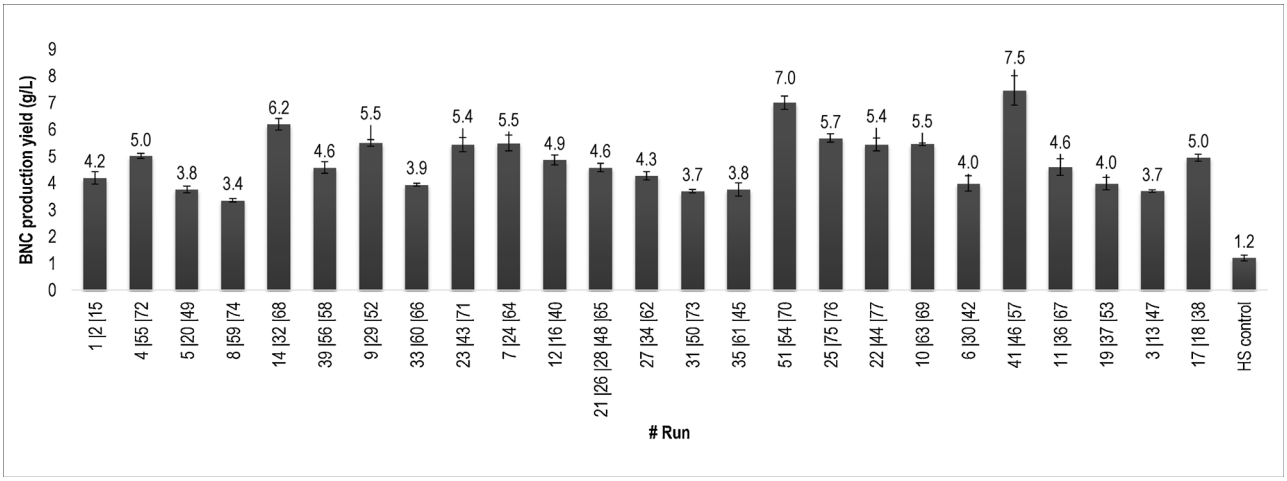

Fig. 1. Experimental BNC production yield using different medium formulations after 9 d, 30 °C in static conditions. Bars with standard deviations represent the means of triplicate experiments.

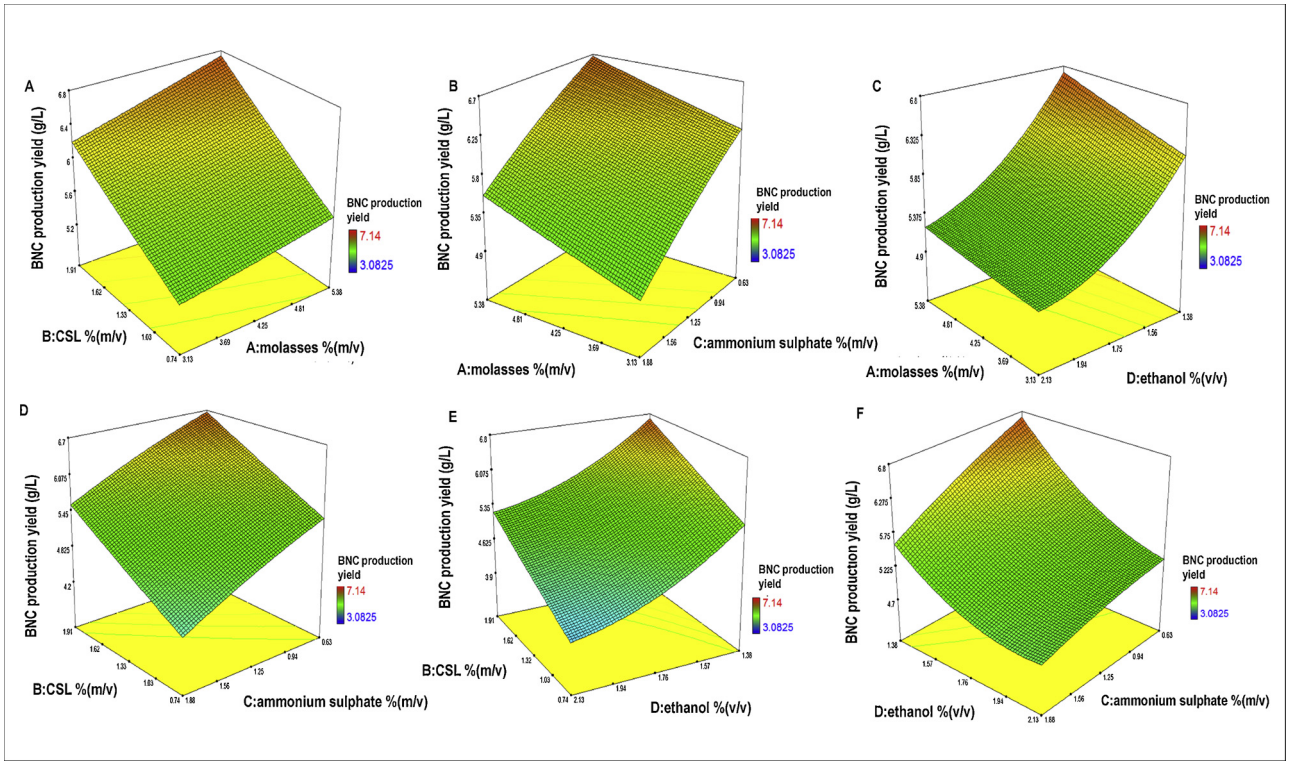

Fig. 2. Response surface curves for BNC production yield. Effect of: **(A)** CSL and molasses; **(B)** molasses and ammonium sulphate; **(C)** molasses and ethanol; **(D)** CSL and ammonium sulphate; **(E)** CSL and ethanol; **(F)** ethanol and ammonium sulphate, on BNC production yield.

the quadratic regression used to produce a second order model was significant, as revealed from the *p*- and *F*-values: the calculated Model *F*-value of 29.56 and the *p*-value of < 0.0001 indicate that the model is significant (i.e. there is only 0.01% probability that the value of "Model *F*-Value" is due to noise). The "Lack of Fit-F-Value" of 2.81 indicates that the Lack of Fit is significant. There is only a 0.74% chance that a "Lack of Fit F-value" this large could occur due to noise. Also, a significant lack of fit (0.0074, Table 4) suggests that there may be some systematic variation, unaccounted for in the hypothesized model. This may be due to the exact replicate values of the independent variable in the model that provide an estimate of pure error.

The second–order polynomial equation of the model fitted for BNC production before eliminating the non-significant terms is:

$$BNC_{production\ yield}\ (g/L) = +\ 12.77 + 0.24 * molasses + 0.30 * CSL - 1.80 * ammonium\ sulphate - 8.06 * ethanol + 0.093 * molasses * CSL + 0.037 * molasses * ammonium\ sulphate - 0.19 * molasses *$$

Table 4
ANOVA analysis of the Response Surface Reduced Quadratic Model, before eliminating the non-significant terms.

	Before eliminating non-significant terms					
Source	Sum of Squares	Df	Mean Square	*F*-Value	*p*-value Prob > *F*	
Model	64.50	14	4.61	29.56	< 0.0001	**Significant**
A-molasses	1.63	1	1.63	10.43	0.0020	Significant
B-CSL	22.90	1	22.90	146.93	< 0.0001	Significant
C-ammonium sulphate	12.89	1	12.89	82.71	< 0.0001	Significant
D-ethanol	19.27	1	19.27	123.64	< 0.0001	Significant
AB	0.18	1	0.18	1.16	0.2851	
AC	0.032	1	0.032	0.20	0.6525	
AD	0.30	1	0.30	1.92	0.1705	
BC	7.191E-003	1	7.191E-003	0.046	0.8306	
BD	9.847E-003	1	9.847E-003	0.063	0.8024	
CD	1.44	1	1.44	9.24	0.0035	Significant
A^2	3.340E-003	1	3.340E-003	0.021	0.8841	
B^2	9.256E-003	1	9.256E-003	0.059	0.8083	
C^2	0.15	1	0.15	0.99	0.3226	
D^2	3.19	1	3.19	20.45	< 0.0001	Significant
Residual	9.66	62	0.16			
Lack of Fit	3.39	10	0.34	2.81	0.0074	Significant
Pure Error	6.27	52	0.12			
Cor Total	74.16	76	4.61	29.56	< 0.0001	Significant
R^2				0.8697		
Adj R^2				0.8403		
Pred R^2				0.7992		
Adeq Precision				18.993		

ethanol + 0.033 * CSL * ammonium sulphate + 0.065 * CSL * ethanol + 0.74 + ammonium sulphate * ethanol + 6.65E-03 * molasses^2 + 0.041 * CSL^2 – 0.15 * ammonium sulphate^2 + 1.85 * ethanol^2 (Eq. 1)

{Degree of freedom = 14; *F*-value = 29.56; *p*-value < 0.0001; R^2 = 0.8697}

Model terms with values of *p*-value Prob > F (Table 4) lower than 0.05 indicated that the model terms are significant; for *p*-value Prob > F higher than 0.1, the model terms are non-significant. In this case, the model terms A (molasses), B (CSL), C (ammonium sulphate), D (ethanol), CD and D^2 were considered significant. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The closer R^2 value to 1.00, the stronger the model is and the better it predicted the observed response. It was suggested that the R^2 value should be at least 0.80, for a good model fitness [54]. Here, the calculated R^2 value of 0.8697 (Table 4), indicated that 13.03% of the total variation could not be explained by the empirical model; this expresses a good enough quadratic fit to navigate the design space. Thus, the response surface model developed in this study for predicting the BNC production may be considered satisfactory. The signal to noise ratio was measured by Adeq Precision value (of 18.993); a ratio greater than 4 also indicates that this model can be used to navigate the design space.

From the above, the second order polynomial equation of the model fitted for BNC production, after eliminating the non-significant terms (Table 4), is:

$BNC_{production\ yield}$ (g/L) = 13.43 + 0.13 * molasses + 0.96 * CSL - 1.97 * ammonium sulphate - 8.99 * ethanol + 0.74 * ammonium sulphate * ethanol + 1.91 * ethanol^2 (Eq. 2)

{Degree of freedom = 6; F-value = 70.70; p-value < 0.0001; R2 = 0.8584}

whereby the F-value increased, meaning that the mean squares of the model are larger than the square residual average. Thus, with a higher the F-value, the more significant *p*-value for ANOVA and the more significant the model is.

The optimal concentrations of the four factors that maximized BNC production yield were predicted using the optimization function of the statistical experimental designs Design Expert 7.1.5. Molasses 5.38% (m/v), CSL 1.91% (m/v) (protein basis), ammonium sulphate 0.63% (m/v), ethanol 1.38% (v/v) were chosen as the optimal concentrations (optimized medium), allowing the highest BNC yield. The predicted medium composition coincided with experiment trials 41, 46 and 57 (Table 3). No statistical differences were observed between the predicted maximum production yield and productivity (6.64 ± 0.4 g/L and 0.737 ± 0.079 g/L/d) and the experimental results (7.5 ± 0.54 g/L and 0.829 ± 0.046 g/L/d) ($p > 0.05$). The optimized results were also confirmed ($p > 0.05$) by conducting a further fermentation experiment in triplicate at the above-optimized values, resulting in a production yield and productivity of 7.6 ± 0.56 g/L and 0.849 ± 0.062 g/L/d, respectively. A Parity plot illustrating the distribution of experimental (actual) and predicted (model) values is shown in Supplementary material, Figure S1. Data points are scattered along the diagonal line, also suggesting that the model is adequate to explain BNC production within the experimental range studied.

Effect of terms on bacterial nanocellulose production

3D response surface graphs (Fig. 2) were plotted to illustrate the interaction of the different paired factors and to determine the optimum of each paired factor for maximum response. Each graph represents the combinations of two test factors in relation to BNC production yield (g/L). The data in Fig. 2 A indicate that the increase in both the carbon (molasses) and protein/nitrogen sources (CSL) resulted in increased BNC production. From the combined effect of molasses and ammonium sulphate concentration (Fig. 2 B), the highest production was obtained with the lowest ammonium sulphate concentration and the highest molasses concentration. Similar results were obtained with the combined effect of molasses and ethanol (Fig. 2 C). The effects of CSL and ethanol concentrations, and of CSL and ammonium sulphate, on BNC production yield are illustrated in Fig. 2 D and E. Yield increased as the concentrations of CSL and ethanol/ammonium sulphate increased and decreased, respectively. The main medium combination of ethanol and ammonium sulphate (CD, Table 4) showed the highest *p*-value for Prob > F (0.0035) and therefore represents a more significant model term combination. Finally, BNC production yield increased with the decrease in ethanol and ammonium sulphate (Fig. 2 F). Ethanol is a well-known carbon source during BNC fermentation and ammonium sulphate is a source of nitrogen [20,49–52]. It is possible that higher concentrations of these nutrients could have led to a substrate growth inhibition and/or affected BNC production. Indeed, Figs. 2 B, C, D and E, where ethanol or ammonium sulphate are present, all show an increase in BNC yield, along with the decrease in these nutrients.

Effect of variable surface area, at constant S/V ratio

Under static culture conditions, due to the aerobic nature of *K. xylinus*, BNC is produced at the air/liquid interface. As synthesis progresses, the extracellular 3D nanofibrillar pellicle accumulates downward into the culture medium, while the metabolically active layer remains at the uppermost interface. In the lower pellicle layer, entrapped cells become inactive or die due to lack of oxygen. BNC yield is known to be dependent on the interplay between surface area and volume of the culture medium and the fermentation time [55]. Previous studies have examined the ratio of surface area to medium volume, attempting to optimize the BNC yield. In one case [26], an optimal surface area/volume ratio of 2.2 cm^{-1} was reported whereas another [20] found that a ratio (S/V) of 0.71 cm^{-1} gave the highest yield using the strain *Acetobacter xylinum* E_{25} and 7 days of fermentation. In addition, it was reported [25] that a ratio of 0.39 cm^{-1} gave the best yield using *Gluconacetobacter xylinus* ATCC 53,524 and 14 d of fermentation.

Here, containers with different areas but with a fixed culture medium depth (2.5 cm) and consequently constant S/V ratio (0.4 cm^{-1}) were used to produce BNC under static culture for 15 d. The amount of BNC dry mass (g) was observed to be directly proportional to the surface area (Fig. 3 A). On the other hand, at a constant S/V ratio of 0.4 cm^{-1}, no statistically significant differences ($p > 0.05$) in production yield (g/L) nor in productivity (g/L/day) were observed between the different surface areas (Fig. 3 B). Accordingly, total sugar consumption (around 72%) and the remaining medium after fermentation (around 17%) were also similar in all assays. These results show that the selected culture medium depth and fermentation time were sufficient to allow the bacteria to produce a BNC pellicle at maximum productivity (Fig. 3 B).

Effect of variable medium depth at constant surface area

When grown under static conditions, a BNC pellicle forms at the air–surface interface. The thickness of this pellicle increases with time, up to a point of stagnation. This is proposed to occur due to oxygen or nutrient limitations: the bacteria across the top layer have poor access to nutrients due diffusional limitations, while those on the bottom layer are deprived of oxygen.

Containers with the same surface area (336 cm^2) were used to evaluate the interplay of these parameters on BNC production yield and productivity, while varying the culture medium depth and fermentation time (Fig. 4). After 9 d fermentation (Fig. 4 A), no statistical differences were observed in the obtained dry mass of BNC using different culture medium depths (1, 2.5 and 4 cm). The same was observed after 15 d for the cultures carried out with a medium depth of 2.5 and 4 cm. BNC production increased linearly with time, until a plateau was reached

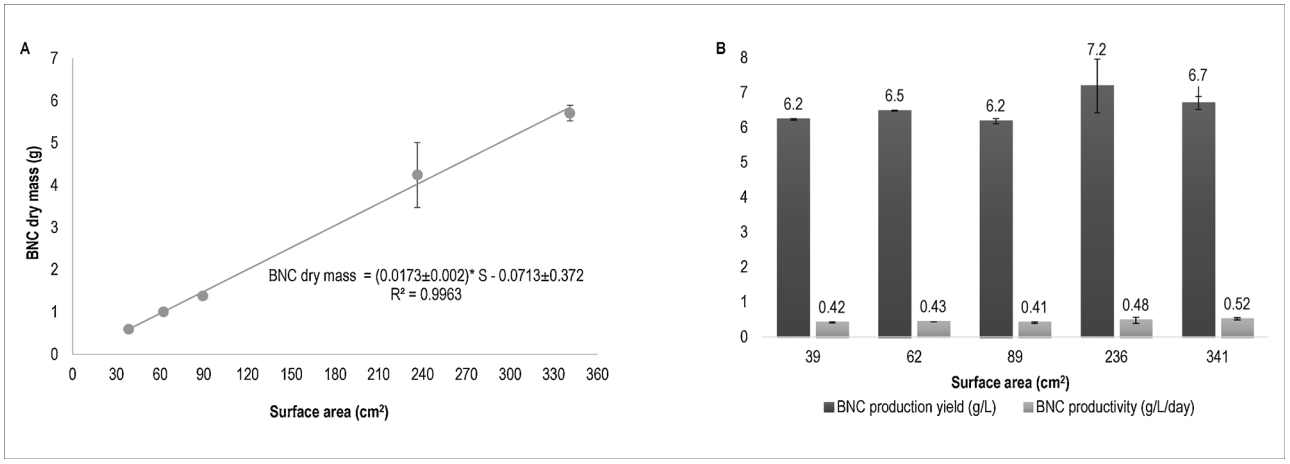

Fig. 3. (A) Relationship between BNC dry mass (g) and surface area (cm^2), after 15 d of static fermentation. **(B)** BNC production yields (g/L) and BNC productivity (g/L/day) obtained using containers with different surface area. after 15 d of static culture. For A and B a fixed culture medium depth of 2.5 cm was used. Bars with standard deviations represent the means of triplicate experiments.

(Fig. 4 B). Decline in production occurred earlier for the cultures with less fermentation medium. For a higher volume (4 cm depth), production progressed linearly until day 21 (Fig. 4 B). The highest BNC production yield (g/L) and productivity (g/L/day) were achieved using a culture depth of 1 cm, as calculated for day 9 (Fig. 4 B and C). In this case, the remaining liquid volume (6% of the initial) and sugars (15% of the initial) were already very low (Fig. 4 D). This suggested that the most efficient production may achieved by maximizing the S/V ratio. Indeed, even higher production yields and volumetric productivities would be reached by further reduction of the culture depth and cultivation time. However, this would not be a feasible alternative for a large-scale static culture of BNC production process, since it would demand a high number of shallow containers and require the frequent replacement of the cultivation vessels (i.e. high cycle times). On the other hand, the BNC mass productivity expressed as g/day, actually increased slightly over time and culture medium depth, as can be concluded by comparing the slopes obtained in Fig. 4 A. Mass productivity for the 4 cm culture depth was higher (0.351 g/day) than those using 1 cm (0.287 g/day) and 2.5 cm (0.296 g/day), possibly due to a slower production rate at the early stage of the fermentation (a lag

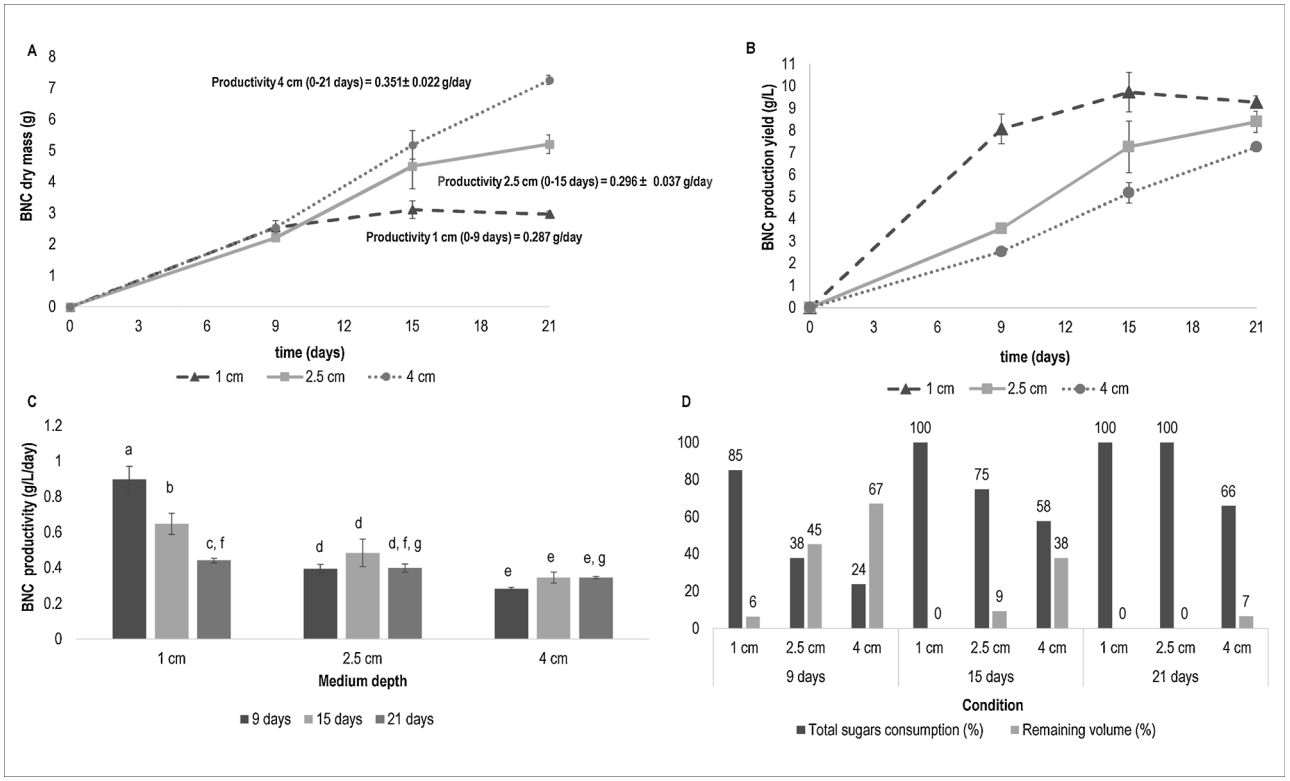

Fig. 4. (A) Relationship between the BNC dry weight (g) and the medium depth (cm) at different fermentation periods. The BNC productivity (g/day) was obtained from the slope of the linear regressions: **(1 cm)** - [0–9 days]; **(2.5 cm) -** [0–15 days] and **(4 cm) -** [0–21 days]. **(B)** BNC production yield (g/L) at different depths, using the same surface area. **(C)** BNC productivity (g/L/day) at different depths, using the same surface area. Different letters between distinct columns denote significant differences using two-way ANOVA ($p < 0.05$). **(D)** Total sugars consumed and remaining volume of culture medium after 9, 15 and 21 d for each tested culture medium depth. Data are presented as average ± standard deviations of experiments run in triplicate.

phase). This could be explained by a lower cell density at early stages, while as the fermentation progressed, for 1 cm culture depth (lower volume), nutrients will have been consumed, limiting productivity. It is important to recall that, for a fixed surface area, cell density is roughly the same, regardless of culture media depth, because bacteria grow at the interface air/liquid. Thus, mass productivity may represent a more relevant parameter of the performance of the static culture fermentation system than the volumetric productivity.

These results demonstrate that, for a culture medium depth of 4 cm, there were no oxygen or nutrient limitations affecting BNC production for up to 21 d, since mass productivity was constant within that time range. In this case, although significant fraction of sugars were still available (34% of the initial), the residual liquid volume was already very low (7% of the initial), hence the BNC production was likely to decline thereafter (Fig. 4 D). Furthermore, these results allow one to plan a cost-effective large scale production of BNC, by equating the volume of fermentation trays and fermentation periods; lower volumes will have shorter cycling times and higher volumetric productivity, but possibly higher operating costs (related to trays discharging, refilling with new fermentation batch and downstream BNC processing), whereas larger volumes may require wider fermentation areas, as the trays will be stored for longer fermentation times, but larger equipment will be required for the downstream processing.

Conclusions

In this study, response surface methodology with central composite design was used to optimize the culture medium formulation for *K. xylinus* BPR 2001, using inexpensive and widely available nutrient sources. Through RSM, the optimum medium composition was % (m/v) molasses 5.38, CSL 1.91 (protein basis), ammonium sulphate 0.63 and ethanol 1.38% (v/v). With this composition, after 9 d static culture fermentation, BNC production yield and productivity were of 6.4 ± 0.54 g/L and 0.74 ± 0.079 g/L/day, respectively. For 15 d fermentation, at a fixed culture media depth, a direct correlation between the fermentation area and BNC dry mass was observed. Also, at a fixed fermentation area, an almost linear BNC productivity of 0.32 ± 0.037 g/L/day, could be maintained for up to 21 d, using a 4 cm culture medium depth. Moreover, for this experimental set up, no nutrient diffusional limitations were observed, the mass productivity being fairly constant overtime.

To date, most studies on BNC production by *K. xylinus* BPR 2001 have used agitated bioreactors and complex culture medium. This work demonstrates that is possible to obtain high yields in static culture, using low cost substrates and a minimal medium composition. This strain and substrates combination should expectably decrease the costs of BNC production.

Conflict of interest

All authors declare that they have no conflict of interest.

Acknowledgements

The authors would like to acknowledge the Portuguese Foundation for Science and Technology (FCT) for the financial support of the PhD grant SFRH/BD/89547/2012 attributed to Ana Cristina Rodrigues, the financial support from project SkinChip: Disruptive cellulose-based microfluidic device for 3D skin modelling, PTDC/BBB-BIO/1889/2014. This study was also supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by European Regional Development Fund under the scope of NORTE 2020 - Programa Operacional Regional do Norte.

The authors acknowledge COPAM Companhia Portuguesa de Amidos, S.A. (Portugal) and RAR Refinarias de Açúcar Reunidas, S.A. (Portugal) for kindly providing CSL and molasses, respectively.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.nbt.2018.12.002.

References

[1] Cleenwerck I, De Wachter M, Gonzalez A, De Vuyst L, De Vos P. Differentiation of species of the family *Acetobacteraceae* by AFLP DNA fingerprinting: *gluconacetobacter* kombuchae is a later heterotypic synonym of Gluconacetobacter hansenii. Int J Syst Evol Microbiol 2009;59(7):1771–86. https://doi.org/10.1099/ijs.0.005157-0.

[2] Kersters K, Lisdiyanti P, Komagata K, Swings J. The family *acetobacteraceae*: the genera *acetobacter, acidomonas, asaia, gluconacetobacter, gluconobacter, and kozakia.* 3 ed.Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. Prokaryotes, 5. New York: Springer; 2006. p. 163–200.

[3] Matsushita K, Inque T, Theeragool G, Trcek J, Toyama H, Adachi O. Acetic acid production in acetic acid bacteria leading to their "death" and survival. In: Yamada M, editor. Survival and death in bacteria. Kerala/India: Research Signpost; 2005. p. 169-18.

[4] Sievers M, Family II Swings J, et al. *Acetobacteraceae.* 2nd ed.Staley JT, editor. Bergey's manual® of systematic bacteriology, 2C. New York: Springer-Verlag New York LLC; 2005. p. 41–94.

[5] Yamada Y, Hoshino K-I, Ishikawa T. Taxonomic studies of acetic acid Bacteria and allied organisms. Part XI. The phylogeny of acetic acid Bacteria Based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *gluconoacetobacter* to the generic level. Biosci Biotechnol Biochem 1997;61(8):1244–51. https://doi.org/10.1271/bbb.61.1244.

[6] Yamada Y, Hosono R, Lisdyanti P, Widyastuti Y, Saono S, et al. Identification of acetic acid bacteria isolated from Indonesian sources, especially of isolates classified in the genus Gluconobacter. J Gen Appl Microbiol 1999;45(1):23–8. https://doi.org/10.2323/jgam.45.23.

[7] Ashori A, Sheykhnazari S, Tabarsa T, Shakeri A, Golalipour M. Bacterial cellulose/silica nanocomposites: preparation and characterization. Carbohydr Polym 2012;90(1):413–8. https://doi.org/10.1016/j.carbpol.2012.05.060.

[8] Saibuatong OA, Phisalaphong M. Novo aloe vera-bacterial cellulose composite film from biosynthesis. Carbohydr Polym 2010;79(2):455–60. https://doi.org/10.1016/j.carbpol.2009.08.039.

[9] Andrade FK, Pertile RAN, Dourado F, Gama FM. Bacterial cellulose: properties, production and applications. In: Lejeune A, Deprez T, editors. Cellulose: structure and properties, derivatives and industrial uses. New York: Nova Science Publishers Inc; 2010. p. 427–58.

[10] Klemm D, Kramer F, Moritz S, Lindström T, Ankerfors M, et al. Nanocelluloses: a new family of nature-based materials. Angew Chem Int Ed Engl 2011;50:5438–66. https://doi.org/10.1002/anie.201001273.

[11] Czaja WK, Young DJ, Kawecki M, Brown RM. The future prospects of microbial cellulose in biomedical applications. Biomacromolecules 2007;8(1):1–12. https://doi.org/10.1021/bm060620d.

[12] de Azeredo HMC. Antimicrobial nanostructures in food packaging. Trends Food Sci Technol 2013;30:56–69. https://doi.org/10.1016/j.tifs.2012.11.006.

[13] Almeida IF, Pereira T, NHCS Silva, Gomes FP, Silvestre AJD, et al. Costa PC. Bacterial cellulose membranes as drug delivery systems: an in vivo skin compatibility study. Eur J Pharm Biopharm 2014;86:332–6. https://doi.org/10.1016/j.ejpb.2013.08.008.

[14] Oliveira Barud HG, Barud H da S, Cavicchioli M, do Amaral TS, de Oliveira Junior OB, et al. Preparation and characterization of a bacterial cellulose/silk fibroin sponge scaffold for tissue regeneration. Carbohydr Polym 2015;128:41–51. https://doi.org/10.1016/j.carbpol.2015.04.007.

[15] Martínez-Sanz M, Lopez-Rubio A, Villano M, Oliveira CSS, Majone M, et al. Production of bacterial nanobiocomposites of polyhydroxyalkanoates derived from waste and bacterial nanocellulose by the electrospinning enabling melt compounding method. J Appl Polym Sci 2016:133. https://doi.org/10.1002/app.42486.

[16] Zhou T, Chen D, Jiu J, Nge TT, Sugahara T, et al. Electrically conductive bacterial cellulose composite membranes produced by the incorporation of graphite nanoplatelets in pristine bacterial cellulose membranes. J Polym Sci Polym Lett Ed 2013;7(9):756–66. https://doi.org/10.3144/expresspolymlett.2013.73.

[17] Esa F, Tasirin SM, Rahman NA. Overview of bacterial cellulose production and application. Agric Agric Sci Procedia 2014;2:113–9. https://doi.org/10.1016/j.aaspro.2014.11.017.

[18] Chawla PR, Bajaj IB, Survase SA, Singhal RS. Microbial cellulose: fermentative production and applications. Food Technol Biotechnol 2009;47(2):107–24.

[19] Lee KY, Buldum G, Mantalaris A, Bismarck A. More than meets the eye in bacterial cellulose: biosynthesis, bioprocessing, and applications in advanced fiber composites. Macromol Biosci 2014;4(1):10–32. https://doi.org/10.1002/mabi.201300298.

[20] Krystynowicz A, Czaja W, Wiktorowska-Jezierska A, Gonçalves-Miskiewicz M, Bielecki S. Factors affecting the yield and properties of bacterial cellulose. J Ind Microbiol Biotechnol 2002;29:89–195. https://doi.org/10.1038/sj.jim.7000303.

[21] Keshk SMAS. Bacterial cellulose production and its industrial applications. J

Bioprocess Biotech 2014;4(02):1–10. https://doi.org/10.4172/2155-9821.1000150.
[22] Shah N, Ul-Islam M, Khattak WA, Park JK. Overview of bacterial cellulose composites: a multipurpose advanced material. Carbohydr Polym 2013;98(2):1585–98. https://doi.org/10.1016/j.carbpol.2013.08.018.
[23] Dourado F, Fontão AI, Leal M, Rodrigues AC, Gama M. process modeling and techno-economic evaluation of an industrial bacterial NanoCellulose fermentation process Chapter 12 In: Gama M, Bielecky S, Dourado F, editors. "Bacterial nanocellulose: from biotechnology to bio-economy"Amsterdam, Netherlands: Elsevier; 2016. p. 199–214.
[24] Jonas R, Farah LF. Production and application of microbial cellulose. Polym Degrad Stab 1998;59:101–6. https://doi.org/10.1016/S0141-3910(97)00197-3.
[25] Ruka D, Simon G, Dean K. Altering the growth conditions of *Gluconacetobacter xylinus* to maximize the yield of bacterial cellulose. Carbohydr Polym 2012;89:613–22. https://doi.org/10.1016/j.carbpol.2012.03.059.
[26] Joris K, Biliet F, Drieghe S, Brack D, Vandamme E. Microbial production of β-1,4-glucans. Meded Fac Landbouwwet-Rijksuniv Gent 1990;55:1563–6.
[27] Toyosaki H, Naritomi T, Akira S, Matsuoka M, Tschuida T, Yoshinaga F. Screening of bacterial cellulose-producing acetobacter strains suitable for agitated culture. Biosci Biotechnol Biochem 1995;59:1498–502. https://doi.org/10.1271/bbb.59.1498.
[28] Tonouchi N, Tahara N, Tsuchida T, Yoshinaga F, Beppu T, Horinouchi S. Addition of a small amount of an endoglucanase enhances cellulose production by Acetobacter xylinum. Biosci Biotechnol Biochem 1995;59:805–8. https://doi.org/10.1271/bbb.59.805.
[29] Bae S, Sugano Y, Shoda M. Improvement of bacterial celulose production by addition of agar in a jar fermentor. J Biosci Bioeng 2004;97:33–8. https://doi.org/10.1016/S1389-1723(04)70162-0.
[30] Bae SO, Shoda M. Production of bacterial cellulose by *Acetobacter xylinum* BPR2001 using molasses medium in jar fermentor. Appl Microbiol Biotechnol 2005;67:45–51. https://doi.org/10.1021/bp0498490.
[31] Bae S, Shoda M. Statistical optimization of culture conditions for bacterial cellulose production using box-behnken design. Biotechnol Bioeng 2005;90:20–8. https://doi.org/10.1002/bit.20325.
[32] Reinalti I, Hrymak AN, Margaritis A. Kinetics of cell growth and crystalline nanocellulose production by Komagataeibacter xylinus. Biochem Eng J 2017;127:21–31. https://doi.org/10.1016/j.bej.2017.07.007.
[33] Joseph G, Rowe GE, Margaritis A, Wan W. Effects of polyacrylamide co-acrylic acid on cellulose production by Acetobacter xylinum. J Chem Technol Biotechnol 2003;78:964–70. https://doi.org/10.1002/jctb.869.
[34] Cheng K-C, Catchmark JM, Demirci A. Effect of different additives on bacterial cellulose production by acetobacter xylinum and analysis of material property. Cellulose 2009;16:1033–45. https://doi.org/10.1007/s10570-009-9346-5.
[35] Chao Y, Mitarai M, Sugano Y, Shoda M. Effect of addition of water soluble polysaccharides on bacterial cellulose production in a 50 L airlift reactor. Biotechnol Prog 2001;17:781–5. https://doi.org/10.1021/bp010046b.
[36] 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;68:345–52. https://doi.org/10.1002/(SICI)1097-0290(20000505)68:3<345::AID-BIT13>3.0.CO;2-M.
[37] Chao Y, Sugano Y, Shoda M. Bacterial cellulose production under oxygen-enriched air at different fructose concentration in a 50-liter, internal-loop airlift reactor. Appl Microbiol Biotechnol 2001;55:673–9. https://doi.org/10.1007/s002530000503.
[38] Cheng K-C, Catchmark JM, Demirci A. Effects of CMC addition on bacterial cellulose production in a biofilm reactor and its paper sheets analysis. Biomacromolecules 2011;12:730–6. https://doi.org/10.1021/bm101363t.
[39] Lin S-P, Liu C-T, Hsu K-D, Hung Y-T, Shih T-Y, Cheng K-C. Production of bacterial cellulose with various additives in a PCS rotating disk bioreactor and its material property analysis. Cellulose 2016;23:367–77. https://doi.org/10.1007/s10570-015-0855-0.
[40] Bae S, Shoda M. Bacterial cellulose production by fed-batch fermentation in molasses medium. Biotechnol Prog 2004;20:1366–71. https://doi.org/10.1021/bp0498490.
[41] Al-Abdallah W, Dahman Y. Production of green biocellulose nanofibers by *Gluconacetobacter xylinus* through utilizing the renewable resources of agriculture residues. Bioprocess Biosyst Eng 2013;36:1735–43. https://doi.org/10.1007/s00449-013-0948-9.
[42] Dahman Y, Jayasuriya KE, Kalis M. Potential of biocellulose nanofibers production from agricultural renewable resources: preliminary study. Apply Biochem Biotechnol 2010;162(6):1647–59. https://doi.org/10.1007/s12010-010-8946-8.
[43] Hu Y, Catchmark JM. Influence of 1-methylcyclopropene (1-MCP) on the production of bacterial cellulose biosynthesized by *Acetobacter xylinum* under the agitated culture. Lett Appl Microbiol 2010;51:109–13. https://doi.org/10.1111/j.1472765X.2010.02866.x.
[44] Zeng X, Small DP, Wan W. Statistical optimization of culture conditions for bacterial cellulose production by *Acetobacter xylinum* BPR 2001 from maple syrup. Carbohydr Polym 2011;85:506–13. https://doi.org/10.1016/j.carbpol.2011.02.034.
[45] Bilgi E, Bayir E, Sendemir-Urkmez A, Hames EE. Optimization Bacterial cellulose production *Gluconacetobacter xylinus* using carob and haricot bean. Int J Biol Macromol 2016;90:2–10. https://doi.org/10.1016/j.ijbiomac.2016.02.052.
[46] Hestrin S, Schramm M. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. Biochem J 1954;58:345–52. https://doi.org/10.1042/bj0580345.
[47] Premjet S, Premjet D, Ohtani Y. The effect of ingredients of sugar cane molasses on bacterial cellulose production by *Acetobacter xylinum* ATCC 10245. Sen-i Gakkaishi 2007;63:193–9. https://doi.org/10.2115/fiber.63.193.
[48] Noro N, Sugano Y, Shoda M. Utilization of the buffering capacity of corn steep liquor in bacterial cellulose production by Acetobacter xylinum. Appl Microbiol Biotechnol 2004;64:199–205. https://doi.org/10.1007/s00253-003-1457-6.
[49] Naromiti T, Kouda T, Yano H, Yoshinaga F. Effect of ethanol on bacterial cellulose production in continuous culture from fructose. J Ferment Bioeng 1998;85:598–603. https://doi.org/10.1016/S0922-338X(98)80012-3.
[50] Son HJ, Heo MS, Kim YG, Lee SJ. Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated Acetobacter sp. A9 in shaking cultures. Biotechnol Appl Biochem 2001;33:1–5. https://doi.org/10.1042/BA20000065.
[51] Park JK, Jung JY, Park YH. Cellulose production by *Gluconacetobacter hansenii* in a medium containing ethanol. Biotechnol Lett 2003;25:2055–9. https://doi.org/10.1023/B:BILE.0000007065.63682.18.
[52] Dourado F, Ryngaillo M, Jedrzejczak-Krzeptowska M, Bielecki S, Gama M. Chapter 1 - Taxonomic Review and Microbial Ecology. in Bacterial Nanocellulose Fermentation. In: Gama M, Bielecky S, Dourado F, editors. "Bacterial nanocellulose: from biotechnology to bio-economy". Amsterdam, Netherlands: Elsevier; 2016. p. 1–17.
[53] Dourado F, Fontão AI, Leal M, Rodrigues AC, Gama M. Process modelling and techno-economic evaluation of an industrial air-lift bacterial cellulose fermentation process Chapter 1 In: Lee Koon-Yang, editor. nanocellulose and sustainability: production, properties, applications, and case studiesUSA: CRC Press; 2018. p. 1–13.
[54] Joglekar AM, May AT. Product excellence through design of experiments. Cereal Food World 1987;32:857–68.
[55] Borzani W, Desouza SJ. Mechanism of the film thickness increasing during the bacterial production of cellulose on non-agitated liquid-media. Biotechnol Lett 1995;17:1271–2. https://doi.org/10.1007/BF00128400.