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Optimizing bacterial nanocellulose production from eucalyptus bark: A circular approach to wastewater management and resource recovery

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ABSTRACT

The production cost of bacterial nanocellulose (BNC) is a major limitation to its widespread use. However, this limitation can be addressed by using alternative low-cost substrates and high-yield strains. Agro-industrial waste-derived substrates offer a cost-effective and sustainable solution, but their high organic load often requires additional downstream wastewater treatments. Here, we optimized static BNC production using eucalyptus bark hydrolysate (EBH) as a low-cost carbon source and proposed a circular approach for wastewater management. Optimization was performed using response surface methodology - central composite design. The optimized EBH medium yielded a 39.7-fold increase compared to standard medium, with a maximum BNC production of 8.29 ± 0.21 g/L. Fermentation wastewater only (WaF) and combined with BNC washing streams (WaW) revealed high levels of organic matter, namely chemical oxygen demand (COD) of 159.0 ± 2.0 and 41.1 ± 0.3 g/L, and volatile solids (VS) of 99.5 ± 0.9 and 26.3 ± 0.2 g/L, respectively, requiring treatment before disposal. A sequential anaerobic-aerobic digestion was investigated for wastewater treatment and valorisation. Anaerobic digestion proved to be effective in treating the wastewater: methanization percentages over 87 % were achieved, and methane productions of 486 ± 2 and 544 ± 30 L/kg VS were obtained from WaF and WaW, respectively. Subsequent aerobic treatment was unsuccessful in further reducing COD levels (approximately 1.5 g/L). Notably, treated wastewater was recycled into the production process up to 45 % without affecting the BNC yield. This study provides valuable insights into the optimization of BNC production from lignocellulosic biomass and the management of wastewater streams, contributing to the development of a more sustainable and economically viable process.

1. Introduction

Bacterial nanocellulose (BNC) is an exopolysaccharide produced by certain gram-negative and strictly aerobic *Komagataeibacter* strains, among others [1–3]. BNC is characterized by its exceptional purity, high crystallinity, and a high degree of polymerization, highlighting its unique physicochemical and mechanical properties. Under static culture conditions, BNC forms an ultrafine fibre network and possesses remarkable water-holding and absorbing capabilities. Additionally, it exhibits remarkable tensile strength even when wet and can be shaped into three-dimensional structures during biosynthesis [4–7]. BNC's biocompatibility and biofunctionality further extend its potential applications. It has been explored in various fields such as drug delivery systems, medical devices, tissue engineering scaffolds, electronic paper displays, leather analogues, textiles, cosmetics and food applications [4, 8–10]. This wide spectrum of potential uses has stimulated interest in the development of innovative approaches to produce BNC on a larger scale. Various fermentation techniques have been tested, including

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agitated, air-lift, membrane, and horizontal bioreactors, employing different fermentation media and engineered mutant strains. However, the high capital and operating costs, coupled with low production yields, have precluded BNC from reaching mainstream [7,11,12].

In many fermentation processes, the cost and availability of substrates play crucial roles in determining the economic feasibility of the process. As a result, there has been an increased focus on using agricultural and industrial wastes as nutrient sources to reduce production costs. These unconventional substrates include fruit juices, vegetable extracts, molasses and syrups, which are not only inexpensive but also serve as rich sources of nitrogen and carbon, such as sugars and alcohols. In some cases, they also provide the necessary cofactors for optimal bacterial growth [13,14]. Some studies have reported increased BNC yield when using these alternative substrates, compared to synthetic culture medium, for example using waste products like apple pomace [15] or corn steep liquor [16], to name a few. The application of different low-cost agro-industrial wastes as alternative media for BNC production, including different optimization strategies for cultivation conditions, has been recently reviewed [17].

The forested regions of Portugal are extensively populated with *Eucalyptus globulus* trees, mostly due to their high commercial importance as a raw material for the pulp and paper industry. As a result, a substantial quantity of eucalyptus-based wastes is produced, including bark (for instance, about 0.5 million metric tons were generated in Portugal in 2017) [18]. Structural carbohydrates typically account for more than half of the total dry weight of eucalyptus bark, making this residue from the pulp industry a cost-effective carbon source for microbial fermentation of biofuels and biochemicals [19,20]. Given the recalcitrant structure of lignocellulosic biomass, the release of its cellulose- and hemicellulose-derived monosaccharides (mainly glucose and xylose) requires, to be fermented, the application of a pretreatment step followed by an enzymatic hydrolysis, resulting in eucalyptus bark hydrolysate (EBH). Steam explosion has been proven to be an effective pretreatment for eucalyptus bark [19,21–23]. Recently, the fermentability of EBH has been demonstrated using yeast for ethanol production [24] and certain *Pseudomonas* strains for polyhydroxyalkanoate biosynthesis [19], suggesting its strong potential. EBH can thus be used as a low-cost carbon source in the preparation of culture media for BNC production.

Despite the advantages of alternative substrates such as EBH, its use generates fermentation waste and wastewater with high organic loads. These must be treated to meet the environmental guidelines and municipal legal limits before disposal. Therefore, integrated pretreatment facilities or dedicated wastewater treatment units are essential for effectively managing the organic load of residues [25]. Despite the capital and operating costs, *in situ* wastewater treatment facilities may offer long-term benefits, including reduced subcontracting costs, the potential for water recirculation and energy generation from wastewater treatment. Biological processes are well-established for industrial-scale wastewater treatment, with anaerobic digestion (AD) being a prominent method. AD reduces the organic load of wastes and produces biogas, a renewable energy source [26,27]. AD is suitable for treating high organic load wastes, including wastewater and solid organic wastes like industrial sludge, food waste, forestry waste, and animal manure [28,29]. It is a multistep process that relies on the coordination of a diverse community of symbiotic microorganisms to convert organic matter into biogas under strict anaerobic conditions [30,31].

The first step of AD consists on the hydrolysis of insoluble or high molecular weight polysaccharides, lipids and proteins; the released monomers and oligomers are then used in the acidogenesis stage to produce volatile fatty acids, ammonia, CO_2, H_2S and other by-products; in the third step, acetogenesis, longer organic acids and alcohols are digested into acetic acid, CO_2 and H_2; the last stage is methanogenesis, where methanogenic archaea convert acetate or H_2 and CO_2 into methane. At the end of this process, the accumulated biogas is composed of 60 – 70 % CH_4, 30 – 40 % CO_2, residual moisture and trace amounts of N_2, H_2 and H_2S [28,31].

AD processes are efficient at handling high organic loads. However, they do not achieve complete mineralization of the organic matter in wastewater, as intermediate organic compounds are usually formed during the process. Moreover, mostly carbon is metabolized, leaving other elements such as nitrogen and phosphate in the digestate, which can be used as land fertilizer [26,27]. One solution to improve the biodegradation of the wastewater from AD is to implement a subsequent aerobic treatment step (activated sludge process), in which the remaining carbonaceous compounds can be further consumed [32,33]. The activated sludge process is widely used in municipal and industrial wastewater treatment. In this process, a suspended bacterial consortium consumes the colloidal and dissolved organic matter in the presence of oxygen, using it for energy and cell growth. Nitrifying autotrophic bacteria oxidize ammonia into nitrate/nitrite; depending on the oxygen concentration, simultaneous nitrification and denitrification can occur, if heterotrophic bacteria can find anoxic conditions to convert the nitrate/nitrite into nitrogen gas. Carbonaceous compounds leftover from the anaerobic stage are also consumed, making aerobic digestion a viable finishing step to complete the removal of the organic load of waste streams [32,33]. The sequential anaerobic and aerobic digestion processes have demonstrated enhanced waste digestibility compared to conventional single-step digestion methods. It provides an additional reduction of the solid and nitrogen (ammonia) content [33,34]. The treated water can be properly disposed of, or it may also be recycled into fresh culture medium (reusing leftover nutrients), enabling cost control and a more eco-friendly and sustainable process.

Given the increasing global demand for alternative functional materials from natural sources, there is a significant opportunity to establish large-scale production facilities of the multipurpose BNC. The main goal of this work is to propose a model for a BNC production unit with considerable self-sustainability. While the production of BNC from agro-industrial wastes and the valorisation of the wastewaters by energy recovery (biogas) have been addressed [25,35], to the best of our knowledge no report has yet shown a fully integrated wastewater treatment and water recycling within the BNC production process. We evaluated, for the first time, the feasibility of an integrated BNC production process by: i) using eucalyptus bark hydrolysate as a carbon source for the BNC fermentation; ii) optimizing the culture medium composition for maximum BNC yield; iii) performing a sequential anaerobic-aerobic treatment of the wastewater from the BNC washing; and iv) exploring the recycling of the treated water back into the BNC production process. This work demonstrates a circular process in which high yields of a biopolymer of increasing commercial interest are obtained from low-cost lignocellulosic biomass and agro-industrial wastes, while recovering and reusing energy, water and nutrients from the wastewater treatment process.

2. Materials and methods

The experimental steps of this study are outlined in Fig. 1.

2.1. Optimization of BNC production from EBH using response surface methodology (RSM) - central composite design (CCD)

2.1.1. Bacterial strain

A *Novacetimonas maltaceti* strain isolated from kombucha (private collection, unpublished data) was used for the BNC fermentation assays. The strain was maintained cryopreserved at (−20 °C and −80 °C) in liquid Hestrin-Schramm culture medium (HS) [36] with 20 % (w/v) of pure glycerol until use.

2.1.2. Preparation of EBH

Eucalyptus bark was collected in the pulp mill of Cacia (Aveiro, Portugal) from The Navigator Company (Portugal). The biomass was pre-treated following a proprietary non-catalyzed steam explosion

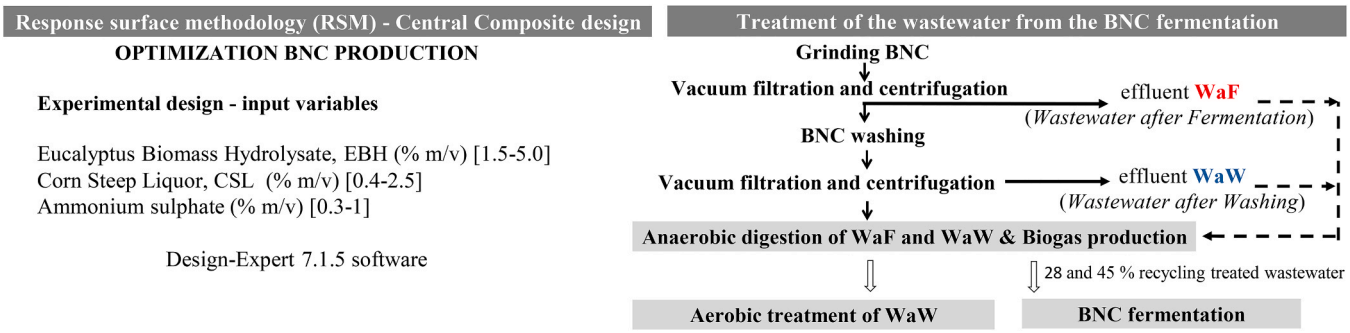

Fig. 1. Schematic representation of the several steps and processing conditions used for the BNC production optimization and treatment of the generated wastewater.

technology, initially developed by the company STEX® (Aveiro, Portugal) and LNEG (Laboratório Nacional de Energia e Geologia, Lisboa Portugal). The steam explosion step was carried out in a 320 L reactor coupled to a 4000 L blow tank where the pre-treated biomass was discharged. The obtained solid fraction was washed with water and subjected to enzymatic hydrolysis. The solid fraction at an initial solids' concentration of 250 g/L (dry basis) was enzymatically hydrolysed at 50 °C in a 600 L stirred tank reactor for 48 h by applying Cellic® CTec3 (Novozymes A/S, Bagsvaerd, Denmark) cocktail at a dosage of 5 % (w/w dry solids). The resulting EBH was centrifuged to remove unreacted solids, frozen at –20 °C and thawed at 4 °C overnight before being used as a carbon source for the BNC production. The released sugars of the EBH were analysed by liquid chromatography (HPLC) according to the procedure described in Section 2.3.

2.1.3. Preparation of the inoculum and static culture fermentation

The *Novacetimonas maltaceti* cells were cultured for 3 days at 30 °C under static conditions in a 1 L conical flask, containing 100 mL of HS medium, initially at a pH of 5.5. The formed cellulose pellicle was vigorously shaken to release the active cells entrapped within the BNC three-dimensional matrix. The cell suspension (3.25 mL – 5 % (v/v) of the final volume) was transferred to a 250 mL beaker with a fermentation area of 33.18 cm^2, containing a final volume of 75 mL of different culture media composition containing EBH, corn step liquor (CSL, a gift from COPAM Companhia Portuguesa de Amidos, S.A.) and ammonium sulphate (AppliChem-Panreac), as detailed in Section 2.1.4. The inoculated media formulations were incubated for 15 days, at 30 °C, under static conditions. As a control, BNC membranes were produced under the same conditions as above but using HS culture medium. Following fermentation, the BNC pellicles were washed with distilled water, at room temperature (RT), to remove the culture medium residues. Subsequently, the pellicles were washed several times with 0.1 M NaOH solution (Fisher-Chemical), also at RT; this solution was changed twice daily, until the membranes turned completely white, as observed by visual inspection. The blanched pellicles were then extensively washed with distilled water, at RT, until the pH became neutral. The purified BNC was oven-dried at 55 °C until it reached a constant mass, and then weighed to determine the BNC production yield (expressed in g of dry BNC/L of culture medium):

$$BNC\ production\ \left(\frac{g}{L}\right) = \frac{dried\ BNC\ (g)}{culture\ medium\ volume\ (L)} \quad (1)$$

2.1.4. Experimental design of the BNC production using response surface methodology (RSM) - central composite design (CCD)

A 2^3 full factorial design was used, with three repetitions at the central point (CP). A total of 17 runs were performed, considering three independent variables (concentrations of EBH, CSL and ammonium sulphate) in the experimental design. The experiment was designed using the Design-Expert 7.1.5 software (Stat-Ease, Inc., USA, Windows operating system). The independent variables were coded as EBH (A) as carbon source, nitrogen from CSL (B) and ammonium sulphate (C), and had three levels: −1, 0, +1. The encoded value 0 corresponds to the center point, whereas the −1 and +1 correspond to the lower and upper values of each variable, respectively (Table 1). The dependent variable was the BNC production yield (Eq. 1). The combinations of the independent variables used in this experimental design are listed in Table 2.

The fermentation experiments were repeated in duplicate, and the mean values and standard deviations are presented. All combinations of the fermentation media were complemented with (% m/v): di-sodium hydrogen phosphate 0.27 and citric acid 0.115. The initial pH of the culture media was adjusted to 5.5 using 15 M NaOH (Fisher-Chemical), and then the medium was autoclaved at 110 °C and 1 bar for 30 min.

The statistical analysis of the experimental design was performed using the Windows version of Design-Expert 7.1.5 software (Stat-Ease, Inc., USA). To evaluate the model, the coefficient of multiple regression R^2 was calculated, showing the model's goodness-of-fit in the data, and the coefficient F of the corresponding analysis of variance (ANOVA) test, which indicates how far the data are scattered from the mean. Three-dimensional plots were drawn to visualize the individual effects and interactions between significant parameters. After selecting the best prediction model, the best combination of the independent variables that led to the maximum response was calculated. This combination was used in a new fermentation assay, and the obtained BNC yield value was compared with that of the predicted yield. Finally, the BNC membrane obtained with the optimal combination of culture medium, which maximized the BNC production, was characterized using the degree of polymerization (DPv), Fourier transform infrared-attenuated total reflection (FTIR-ATR), scanning electron microscopy (SEM) to determine the distribution of the BNC fibres' diameter, and thermogravimetric analysis (TGA). These results have been added to the manuscript as supplementary material.

2.2. Treatment and valorisation of the wastewater from the BNC fermentation

2.2.1. Fermentation and collection of the wastewaters

Static culture fermentations were performed at 30 °C for 45 days, in sterile containers with a surface area of 196 cm^2. The containers were filled with 430 mL of the culture medium with the following composition (% m/v): EBH 5.57 (total sugar); CSL 2.61 (total protein); sodium phosphate dibasic dihydrate 0.339 and citric acid hydrate 0.126. The initial pH was adjusted to 5.5 using 15 M NaOH (Fisher-Chemical) and autoclaved at 110 °C and 1 bar for 30 min. The fermentation medium was then inoculated as described in Section 2.1.3. After fermentation, each wet BNC membrane was weighed, milled for 2 min with a kitchen hand blender and washed, to remove impurities such as culture medium residues and entrapped cells. Each milled BNC membrane was vacuum-filtered and centrifuged at 10,000 rpm for 10 min (Heraeus Multifuge X3R (Thermo Scientific), and the filtrate was stored (this fraction of wastewater obtained after fermentation was designated as WaF). The collected BNC cake was then sequentially washed by multiple steps of

Table 1
Actual limit concentrations and coded factor values (% m/v) of the response surface methodology for the optimization of BNC production.

Sources	Variable	Symbol	Actual lower limit	Actual higher limit	Low coded (-1)	High coded (+1)	Central point (0)
Carbon	EBH (total sugar)	(A)	1.5	5.0	0.31	6.19	3.25
Nitrogen	CSL (total protein)	(B)	0.4	2.5	0	3.22	1.45
	Ammonium sulphate	(C)	0.3	1	0.06	1.24	0.65

Table 2
Experimental central composite design matrix for the three variables.

# Run	A: EBH % m (total sugar)/v	B: CSL % m (total protein)/v	C: Ammonium sulphate % (m/v)
1 (CP)	3.25	1.45	0.65
2	1.5	0.40	1.00
3	3.25	0.00	0.65
4	1.5	2.50	0.30
5	5	0.40	1.00
6	6.19	1.45	0.65
7	3.25	1.45	1.24
8	5	0.40	0.30
9	5	2.50	0.30
10	5	2.50	1.00
11	3.25	1.45	0.06
12 (CP)	3.25	1.45	0.65
13	1.5	2.50	1.00
14	1.5	0.40	0.30
15	3.25	3.22	0.65
16 (CP)	3.25	1.45	0.65
17	0.31	1.45	0.65

CP - central point of the optimization model.

vacuum filtration and centrifugation at 10,000 rpm for 10 min, as follows: a) the BNC cake was resuspended in an aqueous solution of 0.1 M NaOH, making up the initial BNC membrane mass. The mixture was incubated for 15 min (RT), vacuum filtered and centrifuged; b) this process was repeated a second time; c) the BNC cake was then resuspended in an aqueous solution of 4 % (v/v) commercial white wine vinegar, making up half of the initial BNC membrane mass, incubated for 15 min and then vacuum filtered and centrifuged; d) finally, the BNC cake was washed with distilled water (making up the initial BNC membrane mass), incubated for 15 min and then vacuum filtered and centrifuged. The obtained filtrates were combined (designated wastewater after washing – WaW). Both WaF and WaW filtrates were stored at −20 °C. Before use, the wastewaters were thawed, and WaW was neutralized with white wine vinegar to a pH between 6.8–7.2, reported the ideal range for methanogenic archaeal growth [37].

2.2.2. Anaerobic treatment of the BNC wastewaters

Anaerobic treatment of the BNC production wastewaters was performed in batch assays, using 1100 mL glass bottles with a working volume of 100 mL. The anaerobic inoculum consisted of a 50/50 % mixture (m/m of volatile solids, VS) of an anaerobic granular sludge, from a brewery wastewater treatment plant, and a suspended sludge from a municipal wastewater treatment plant. This mixture was incubated at 37 °C for 2 days, to deplete the residual substrate (degassing process) prior to the assays. The VS' content of the inoculum was 38 ± 3 g/kg, determined by standard methods [38,39]. A volume of 55 mL of inoculum was added to the bottles (final VS concentration of 20.7 g/L), as well as BNC wastewaters as substrate (VS of 5.2 g/L), that were diluted with buffer medium to make up an inoculum to substrate ratio of 4.0 (VS/VS). Therefore, to have the 5.2 g/L of VS, in the beginning of the assay WaF and WaW were added in a final concentration of COD of 8.3 g/L and 8.1 g/L, respectively. The buffer medium was prepared according to Holliger et al. [40], and supplemented with salts, vitamins, trace metals and selenite, as described by Angelidaki and Sanders [41]; sodium bicarbonate (3 g/L) (JMGS) was also added to ensure the buffered conditions. The bottles were closed with rubber stoppers and aluminium caps and the headspace was flushed with a mixture of nitrogen/carbon dioxide (N_2/CO_2, 80/20 % v/v) at a final pressure of 1.1 bar. Finally, the bottles were reduced with sodium sulfide nonahydrate (Acros organics) to a final concentration of 0.025 % (m/v).

In parallel, a blank assay (without substrate) was made to estimate the background methane produced by the inoculum, and a positive control was also prepared with 0.52 g of microcrystalline cellulose (Acros organics) as substrate, to validate the performance of the inoculum. All assays were performed in triplicate, and incubated at 37 °C, with manual agitation once a day.

During incubation, the accumulated methane was quantified by gas chromatography. The cumulative methane production over time was expressed as the volume of methane at standard temperature and pressure conditions (0 °C and 1.013 bar) [42] per amount of VS of substrate added (L/kg), after discounting the methane production in the blank assay. The percentage of methanization (PM) was determined with the following equation:

$$PM\ (\%) = (COD_{CH4})/(COD_{initial})\ x\ 100 \quad (2)$$

where COD_{CH4} represents the chemical oxygen demand (COD, g) converted to methane (subtracting the methane of the blank assay), and $COD_{initial}$ is the COD (g) of the substrate in each bottle at the beginning of the assay ($COD_{initial}$ was 0.83 g in WaF bottles and 0.81 g in WaW bottles).

At the end of the assays, the content of each bottle was centrifuged for 20 min at 11,000 rpm (Heraeus Multifuge X3R, Thermo Fisher Scientific) to separate the biomass, and the digested wastewaters (WaF_AD, WaW_AD) were characterized in terms of pH, COD, total nitrogen (TN) and ammonium, as described in Section 2.3.

2.2.3. Aerobic treatment of the BNC wastewaters after anaerobic digestion

The inoculum for the aerobic treatment consisted of an activated sludge from a municipal wastewater treatment plant, with a VS concentration of 9.8 g/kg. The assays were performed in 250 mL Erlenmeyer flasks with cotton plugs, using a working volume of 50 mL. The inoculum was added to each flask to attain a VS of 3 g/L (15.4 g), and the remaining volume was made up with wastewater from the anaerobic digestion of WaW_AD). In parallel, using the same amount of inoculum, control tests were made with: (CTR A) WaW_AD diluted (1:2) with phosphate buffer (50 mM, pH 7); (CTR B) WaW_AD diluted with phosphate buffer and supplemented with 0.5 g/L sodium acetate; (CTR C) WaW_AD diluted (1:2) with distilled water and supplemented with 0.5 g/L acetate; (CTR D) positive control without WaW_AD and with 0.5 g/L acetate (in distilled water). The flasks were incubated at RT with orbital agitation at 150 rpm. Total COD (CODt) and pH values were monitored over time.

2.2.4. BNC production with treated wastewater

To evaluate the potential of recycling the treated wastewater, WaF_AD and WaW_AD were used in the preparation of the culture medium for a new BNC fermentation. Both wastewaters were tested at two concentrations, 28 and 45 % (v/v), the highest concentration representing the maximum incorporation possible considering that EBH and CSL are both present in liquid form. The composition of the obtained

culture medium was, for 28 % of treated wastewater in (% m/v): EBH (total sugar) 5.57; CSL (total protein) 2.61; Na_2HPO_4 0.27 and citric acid 0.115; for 45 % of treated wastewater in (% m/v): EBH (total sugar) 4.0; CSL (total protein) 2.36; Na_2HPO_4 0.27 and citric acid 0.115. In parallel, for each condition, the treated wastewater was replaced with distilled water, as control (CTR). The initial pH of the culture medium was adjusted to 5.5 using 15 M NaOH (Fisher-Chemical) and autoclaved at 110 °C, 1 bar for 30 min. The static culture fermentations were done for 15 days at 30 °C in a 250 mL beaker with a fermentation area of 33.18 cm^2 containing 75 mL of medium. Afterwards, the BNC pellicles were purified as previously described, oven-dried at 55 °C until constant mass and weighed to calculate the BNC production yield (g/L).

2.3. Analytical methods

The composition of EBH and the residual sugars at the end of fermentation time were determined by HPLC (LC 2060 C, Shimadzu, Japan), using an Aminex HPX-87H column (300 mm × 7.8 mm, particle size 8 μm) coupled to RI and UV detectors under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.6 mL/min, and column temperature 25 °C. The injection volume was 10 μL. The concentrations of glucose, xylose and fructose were determined based on calibration curves obtained using pure compounds.

The total protein content in CSL was (% m/v) 15.8 ± 0.6, as determined by using a BCA protein assay kit (Pierce® BCA 23227 Protein Assay Kit, Thermo Scientific).

The pH and salinity values were measured with a benchtop multiparameter analyser (model C3010, Consort, Turnhout, Belgium). Total solids (TS, the residue left after drying the sample at 105 °C) and volatile solids (VS, the weight loss after a sample is heated to dryness and ignited at 550 °C) were determined according to Standard Methods [38,39]. COD, TN, ammonium (NH_4^+), sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}) levels were determined using standard kits (Hach Lange, Düsseldorf, Germany). To determine the CODt, the samples were directly analysed, while for soluble COD (CODs), samples were previously centrifuged for 10 min at 20 000 rcf (Eppendorf 5430 R, rotor F-35–6–30) and filtered through a 0.22 μm PES syringe filter; for soluble plus colloidal COD (COD(s + c)), the samples were centrifuged for 10 min at 20 000 rcf (not filtered), and the supernatant was collected and analysed.

Methane was quantified on a GC-2014 Shimadzu ATF model equipped with a Porapak Q column (80–100 mesh) (2 m x 3.75 mm) with a FID detector and a flow rate of 30 mL min^{-1} of N_2 as carrier gas. The temperatures of the detector, injector and oven were of 220 °C, 110 °C and 35 °C, respectively. A sample volume of 500 μL was injected with a gas-tight syringe.

All parameters were measured in triplicate and are presented as the mean and standard deviation.

2.4. Statistical analysis

One-way and two-way ANOVA were performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Optimization of the BNC production

3.1.1. Response surface methodology – central composite design

Response surface methodology (RSM) is a multivariate technique that combines mathematical and statistical tools to analyse and model complex relationships between multiple independent variables and the responses they produce on a dependent variable, allowing statistical predictions to be made. Central composite design (CCD) is a widely used methodology in which the upper and lower limits of each factor are set for an experimental design, allowing the identification of statistically significant interactions between the variables and the combination of factors that generate a particular optimal response [43,44].

In this work, RSM-CCD was used to optimize BNC production yield, using three independent variables (EBH, CSL and ammonium sulphate). The results obtained showed a strong correspondence between the experimental and predicted values (calculated using the regression equation), as shown in Table 3. The final concentration of BNC varied between 2 and 8 g/L, depending on the composition of the culture medium. The set of optimal statistical conditions, runs CP, 9, 10, and 11, correspond to the conditions with the highest BNC yield, with no significant differences observed among them ($p > 0.05$, one-way ANOVA and Bonferroni test). Thus, a mean value for the BNC production yield of 7.20 ± 0.97 g/L was calculated. Under these conditions, more than 90 % of the initial sugars were consumed. The EBH composition (% m/v) was determined as: 90 % total sugars, from which glucose 8.5–9.0 and xylose 1.0–1.5, acetic acid 0.5, formic acid 0.4, levulinic acid, furfural and hydroxymethyl furfural (HMF) were present in residual amounts. The residual sugars at the end of the fermentation consisted mostly of xylose, namely a mean of 0.33 ± 0.09 % of glucose and 7.3 ± 1.7 % of xylose of the initial sugars. The mean value of BNC production here obtained with EBH represents around a 34.5-fold increase in BNC yield as compared with the HS medium (Fig. S1, supplementary material), demonstrating the positive impact of the alternative and complex substrates of the EBH medium over a partially or totally synthetic one on the BNC yield. Also, the BNC production here achieved, obtained using a simplified and low-cost culture medium, was similar to or higher than that reported in the literature using other forest/lignocellulosic biomass wastes as carbon sources for BNC production. For example, in a study with *Komagataeibacter rhaeticus K3*, using the same type of substrate (EBH) [45], the maximum experimental BNC yield was of 5.46 g/L. Other examples where lignocellulosic biomass was also used include: aspen hydrolysate (2.9 g/L BNC) [46], elephant grass hydrolysates (6.4 g/L BNC) [47], palm fronds hydrolysate (3.9 g/L BNC) [48], spruce wood hydrolysate (8.2 g/L BNC) [49], and poplar wood sawdust hydrolysate (3.14 g/L BNC) [50]. The strains used in these studies were *Komagataeibacter xylinus* B-12429, *Komagataeibacter xylinus* CH001, *Bacillus velezensis* SMR, *Komagataeibacter xylinus* ATCC 23770, and *Komagataeibacter xylinus*, respectively.

The regression equation that describes the dependence of the response (BNC yield) of the variables (EBH, CSL and ammonium sulphate) was obtained by fitting the experimental data to a quadratic model. The statistical significance of the quadratic polynomial model

Table 3
Experimental and predicted values of the BNC production yield using different medium formulations, after 15 days at 30 °C in static conditions.

	Predicted	Experimental	
# Run	**BNC yield (g/L)**	**BNC yield (g/L)**	**Consumed sugars (%)**
1 (CP)	6.38	6.00 ± 0.03	90.5
2	2.75	3.0 0 ± 0.02	91.8
3	0.13	0.53 ± 0.1	93.4
4	4.37	4.87 ± 0.23	68.7
5	2.58	2.14 ± 0.78	90.1
6	5.85	6.08 ± 0.63	92.5
7	6.54	6.35 ± 0.01	90.7
8	3.30	3.20 ± 0.60	85.1
9	8.37	8.17 ± 0.10	94.6
10	7.86	8.26 ± 0.24	92.9
11	7.50	7.60 ± 0.06	91.3
12 (CP)	6.38	6.93 ± 0.01	91.3
13	3.94	4.11 ± 0.32	63.9
14	3.38	3.05 ± 0.28	80.3
15	5.40	4.91 ± 1.04	78.2
16 (CP)	6.38	6.23 ± 0.06	89.7
17	2.63	2.30 ± 0.82	49.7
HS medium	—	0.21 ± 0.04	95.8

CP - central point of the optimization model.

equation was evaluated by conducting an ANOVA analysis (Table 4). Results show a high significance for the design results with an *F*-value of 32.99 and a significance *F*-value of < 0.0001, which indicates that there is only a 0.01 % chance that a value of the "Model F-Value" this large could occur due to noise. Further, regression analysis (Fig. 2 A) showed a significant consistency between the experimental and predicted responses in the BNC yield, as the calculated R^2 was 0.9770 and the adjusted-R^2 was 0.9474 (Table 4). The consistency of the CCD results is indicated by the closeness of the R^2 value to 1 [51], the result from the adjusted-R^2 also indicating that > 95 % of the model results are attributed to the three studied variables [52]. Moreover, the "Pred R-Squared" (that indicates the model's ability to predict the dependent variable) of 0.8467 is in reasonable agreement with the "Adj R-Squared" (that adjusts for the number of predictors in the model to avoid overfitting) of 0.9474, indicating that the model is not overfitting the data. Finally, the signal to noise ratio, as determined by the "Adeq Precision" value, was greater than 4 (20.449), which indicates an adequate signal and that this model can be used to navigate the design space.

Regarding the lack of fit test, the F-value compares the variance between the "model not fitting" (lack of fit) to the variance of the replicated points (pure error). The "Lack of Fit F-value" of 1.25 and the p-value Prob >F of 0.44997 imply that the lack of fit is not significant relative to the pure error, meaning that the observed variation is within the expected range and is not attributable to the model's insufficiency. Therefore, there is a 49.97 % chance that a "lack of fit F-value" could occur due to noise or random variation, rather than issues with the model's appropriateness or form. Since the *p*-value was larger than the chosen significance level (α 0.5), the model was appropriate for the data.

For the model terms "A-EBH", "B-CSL" and "C-ammonium sulphate", the values of "Prob > F" < 0.05 indicate that the model terms are significant (S). Values with "Prob > F" > 0.1 indicate that the model terms are nonsignificant (NS). Values within the range 0.05 < "Prob > F" < 0.1 indicate that the terms are borderline significant (BS) or marginally significant to the model, as they possess a higher likelihood of contributing to the model compared to nonsignificant terms. Eliminating the nonsignificant terms (AC, BC, C^2) further improves the model's precision.

The surface plot elucidates the effect of the interaction between two independent variables (represented on the X-axis and Y-axis) on the dependent variable BNC production yield (represented on the Z-axis), while the others remain constant and at their middle value [16]. The surface plots are shown in Fig. 2 (B–D). The results show that increasing the concentrations of EBH and CSL led to an increase in the BNC yield (Fig. 2B); the highest BNC yield was obtained with approximately EBH 5 % (m/v) and a minimum concentration of ammonium sulphate (Fig. 2 C), and finally, higher BNC production yields occurred at the lower ammonium sulphate concentrations and higher CSL concentrations (Fig. 2D). From this comparative analysis, the CSL and EBH terms were the most relevant combination affecting the BNC yield (lowest p-value Prob > F (0.0009) (Table 4).

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

Source	Sum of squares	Df	Mean square	*F*-value	*p*-value Prob > *F*	
Model	81.93	9	9.10	32.99	< 0.0001	S
A-EBH	12.54	1	12.54	45.46	0.0003	S
B-CSL	33.50	1	33.50	121.41	< 0.0001	S
C-ammonium sulphate	1.11	1	1.11	4.02	0.0849	BS
AB	8.32	1	8.32	30.17	0.0009	S
AC	3.52E−03	1	3.56E−03	0.013	0.9127	NS
BC	0.022	1	0.022	0.079	0.7862	NS
A^2	6.47	1	6.47	23.46	0.0019	S
B^2	18.44	1	18.44	66.84	< 0.0001	S
C^2	0.57	1	0.57	2.07	0.1931	NS
Residual	1.93	7	0.28			
Lack of Fit	1.46	5	0.29	1.25	0.4997	NS
Pure Error	0.47	2	0.23			
Cor Total	83.86	16				
R^2				0.9770		
Adj R^2				0.9474		
Pred R^2				0.8467		
Adeq Precision				20.449		

S - Significant; NS - Nonsignificant; BS - Borderline significant.

From the above, the design model was expressed as a second-order linear regression equation, after eliminating the nonsignificant terms:

$$BNC_{production}\left(\frac{g}{L}\right) = 0.46187 + 1.49236 \times EBH + 3.22683 \times CSL - 0.81462 \times ammonium\ sulphate + 0.55512 \times EBH \times CSL - 0.26917 \times EBH^2 - 1.22047 \times CSL^2 \quad (3)$$

(Degrees of freedom = 6; *F*-value = 53.61; *p*-value < 0.0001; R^2 = 0.9698)

whereby the *F*-value increased (as compared to Table 4), translating to a more significant model.

After numerical optimization using the statistical experimental designs of Design Expert, the calculated concentrations of the three factors that maximized the BNC production yield were (% m/v): EBH 5.57 (total sugar), CSL 2.61 (total protein), ammonium sulphate 0.0 with a predicted BNC yield of 8.91 ± 0.69 g/L, with 95 % Cl low = 7.30 and 95 % Cl high = 10.51 (Fig. 3). These conditions were proximal to those of Run 9 (Table 2).

3.1.2. Validation of the CCD model predicted results

To evaluate the consistency of the model, new fermentation assays were done using the calculated optimum conditions and RUN 9 condition (Table 2). These assays resulted in an experimental BNC production yield of 5.91 ± 0.33 g/L for the calculated optimum conditions, below the model's predicted value (of 8.91 g/L); 8.29 ± 0.21 g/L for the RUN 9 condition, that represents a 39.7-fold increase compared to standard medium, close to the previously obtained values (Table 3).

The concentrations under the optimal conditions were not significantly different from those used in the RUN 9 condition. The absence of ammonium sulphate in the predicted optimal conditions by the model may have had an unforeseen impact on the fermentation process and BNC yield. This absence may indicate potential metabolic or physiological shifts not accounted for in the model. Ammonium sulphate is a readily available nitrogen source, essential for protein synthesis, including enzymes involved in cellulose production. It can contribute to the osmotic balance of the fermentation broth, helping to regulate the water potential and maintain cell membrane integrity. Alternative nitrogen sources (e.g., from CSL or from EBH) may have been insufficient or excessive, reducing microbial growth. Under nitrogen limitation or excess, bacteria may shift their metabolism, or, under osmotic imbalance, divert energy from BNC production to maintain cellular homeostasis [53–58]. Collectively, these effects may have affected the BNC production. Despite this single discrepancy, the model can be used to navigate the design space.

The BNC membrane produced under the optimal condition RUN 9 was characterized by the degree of polymerization (DPv), functional chemical groups (FTIR), surface morphology and diameter size distribution (SEM), and thermal stability (TGA). The results obtained are presented in more detail in the supplementary material. The characterization of the BNC indicated that the EBH had no impact on its physical and chemical features, which might play an important role in the possible application phase. The determined degree of polymerization was of 2422 ± 75 (corresponding to a molecular weight (Mw) of 392 ± 12 kDa). The diameters of the nanofibers present a mean value of 38.03 ± 8.2 nm and high thermal stability (DTG peak temperature at 374 °C). With these results we can assume that the inconsiderable

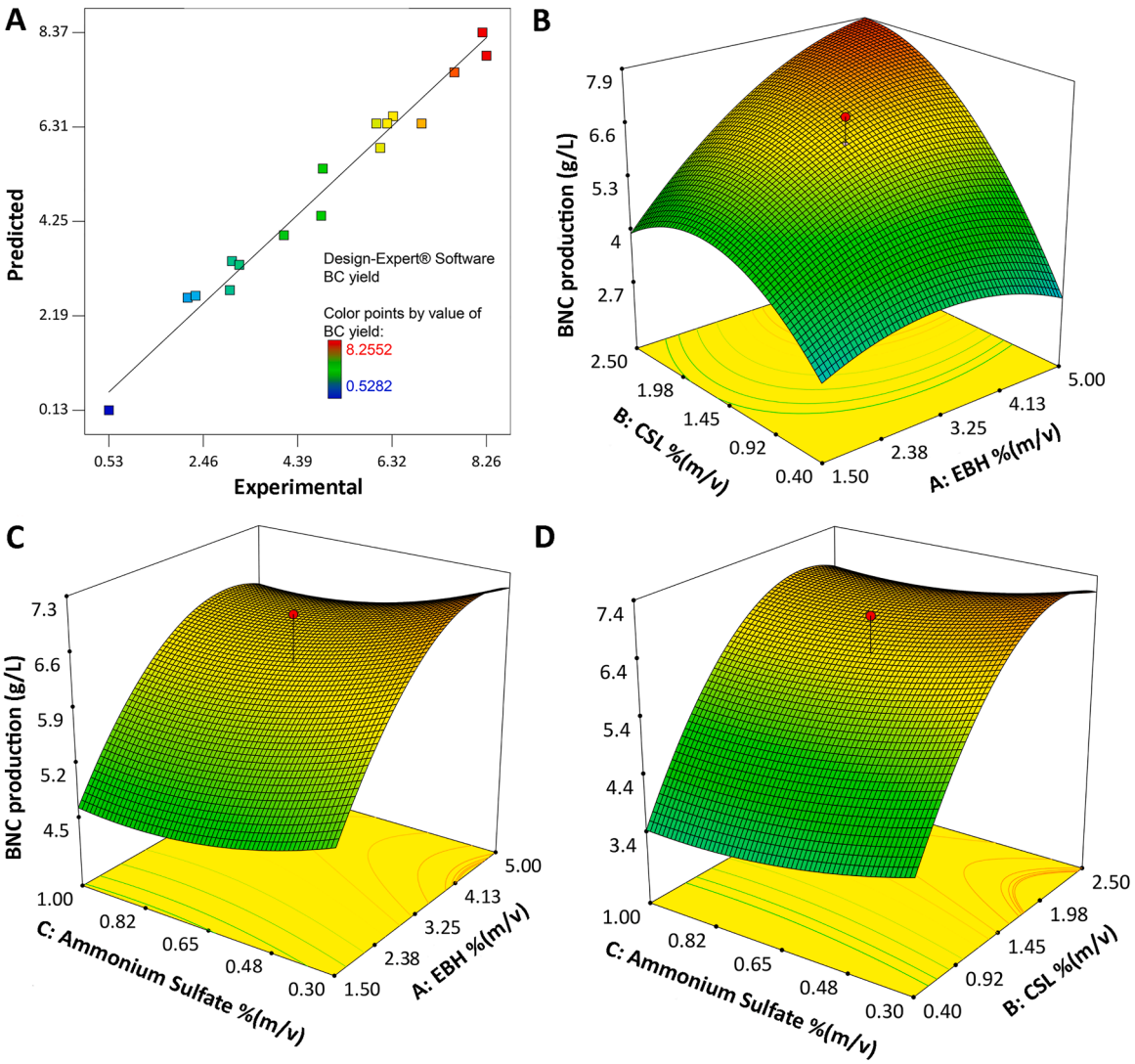

Fig. 2. (A) Parity plot of predicted values against experimental data of BNC yield according to the experimental design; 3D-imaging of the BNC production response surface as a function of CSL and EBH (B), EBH and ammonium sulphate (C), CSL and ammonium sulphate (D).

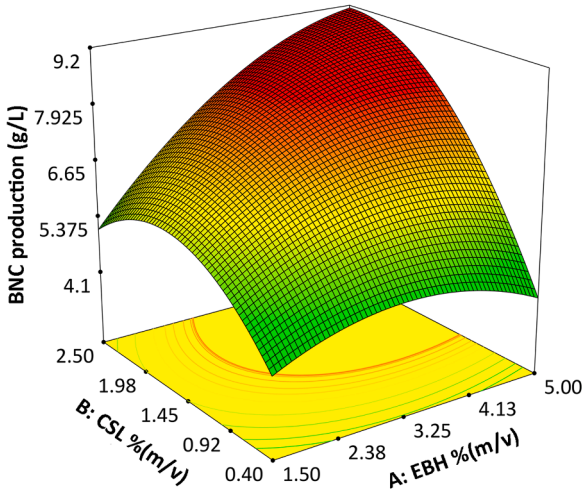

Fig. 3. 3D representation of the BNC production yield as a function of the concentration of EBH and CSL, using the experimental statistical model's optimization function in Design Expert 7.1.5.

impact of the eucalyptus wastes on the microstructural and morphological features of the produced BNC nanofibers make EBH an effective substitute for pure D-glucose.

3.2. Treatment and valorisation of the wastewater from the BNC fermentation

3.2.1. Characterization of the wastewaters

Following optimization of the culture medium, new BNC fermentation assays were done, to collect the wastewater for a sequential anaerobic-aerobic treatment. Both WaF and WaW were dark coloured liquids with suspended solid particles. The characterization of these wastewaters is presented in Table 5.

The pH of WaF was 6.9, whereas WaW had a pH of 9.3, resulting from the washing steps with NaOH. This waste stream had to be neutralized (to 7.2) before anaerobic digestion, in order to match the optimal pH range for methanogenic archaea [37]. Expectably (Table 5), WaW was more diluted than WaF in all measured parameters.

In the particular case of the Portuguese legislation (Annex XVIII from the decree-law 236/98, 1st of August), discard limits for wastewaters include, among other parameters, COD < 150 mg/L, TN < 15 mg/L, TS < 60 mg/L and sulphates < 2.0 g/L. Industrial wastewaters can also be discarded to a public collector to be treated by municipal entities; in this

Table 5
Characterization of the WaF and WaW wastewaters before the anaerobic treatment.

	WaF	WaW
pH	6.9	7.2
Salinity (g/L)	16.0 ± 0.1	8.0 ± 0.0
TS (g/L)	130.0 ± 0.1	42.1 ± 0.1
VS (g/L)	99.5 ± 0.9	26.3 ± 0.2
CODt (g/L)	159.0 ± 2.0	41.1 ± 0.3
CODs (g/L)	113.2 ± 11.8	35.9 ± 0.7
TN (g/L)	8.2 ± 0.1	2.1 ± 0.1
NH_4^+-N (g/L)	1.0 ± 0.0	0.1 ± 0.0
NH_4^+ (g/L)	1.3 ± 0.0	0.2 ± 0.0
SO_4^{2-} (g/L)	4.3 ± 0.1	1.1 ± 0.1

case, for example in the city of Braga (Portugal), the discard limits are CQO < 1000 mg/L, TN < 40 mg/L, TS < 1000 mg/L and sulphates < 2 g/L (under Regulation No. 169/2015, published in Diário da República, 2nd series — No. 71 — April 13, 2015). However, both WaF and WaW exhibit higher values and require treatment prior to disposal. It is worth mentioning that these wastewaters also had a high concentration of salts, free ammonia and sulphates, which are known to have inhibitory effects on anaerobic digestion when at high levels [28,29].

3.2.2. *Anaerobic digestion of the wastewaters*

In this work, WaF and WaW were first diluted in the anaerobic assay to guarantee an inoculum-to-substrate ratio of 4 (VS/VS) (see Section 2.2.2), this way decreasing the concentration of inhibitory compounds potentially present in the wastewaters. Fig. 4 displays the cumulative methane produced over time from the anaerobic digestion of WaW and WaF wastewaters, after subtracting the production in the blank assays, and Table 6 shows the efficiency parameters of the process.

From Fig. 4, no lag phase seems to have occurred. CH_4 production started immediately, reaching at least 70 % of its maximum after 2 days, for both waste streams, gradually slowing down until reaching a plateau, around 486 ± 2 L/kg VS from WaF, and 543 ± 30 L/kg VS from WaW (corresponding to 304 ± 1 L/kg COD and 348 ± 19 L/kg COD, respectively). The batch assays were stopped after 21 days, when meeting the BMP assay finishing criteria, such as daily methane production during three consecutive days being < 0.5 % of the total accumulated volume of methane [25,40]. Thus, fairly high methane yields were obtained from these wastewaters, taking as reference methane production values

Table 6
Maximum cumulative methane production obtained from WaF and WaW streams, percentage of methanization (PM) and COD(s + c) of the BNC wastewaters after anaerobic digestion (WaF_AD and WaW_AD).

	WaF	WaW
Maximum cumulative methane production (L/kg VS)	486 ± 2	544 ± 30
Maximum cumulative methane production (L/kg wastewater)	48 ± 0	14 ± 1
PM (%)	87 ± 0	99 ± 6
	WaF_AD	**WaW_AD**
COD(s + c) (g/L)	1.4 ± 0.0	1.5 ± 0.0

obtained in batch assays from different waste sources: 313 L/kg COD from fishery wastewaters [59]; 116 and 323 L/kg VS from wood and pulp residues, respectively [60]; 411 L/kg VS from brewery by-products [61] or 440–1400 L/kg VS from food waste [62].

From Table 5, soluble COD (CODs) accounted for 87 % of the total COD in WaW, and 71 % in WaF, meaning that the former contains more solubilized organic matter. Most of the residues in WaW were disintegrated following washing with NaOH. Alkaline treatment is in fact one of the advised waste pretreatments before anaerobic digestion, to facilitate the dissolution of organic matter [63]. This could explain the higher methane production (per kilogram of VS) and higher PM (almost at 100 % of the theoretical value) obtained with WaW (Table 6). With the same initial VS and COD, WaW digestion is thus more efficient; however, as WaF is more concentrated, i.e., it presents higher values of COD and VS, there is a greater yield of CH_4 per kilogram of wastewater. Importantly, this assay highlighted the feasibility of using AD for the valorisation of the wastewaters from the BNC fermentation process, through the production of biogas, which can be converted into energy for the BNC process. This may represent an important cost reduction when considering an industrial production facility [11,25].

At the start of the anaerobic assays, the COD was of 8.3 g/L for WaF and 8.1 for WaW. At terminus, despite the major content of COD had been converted to methane (87 ± 0 % in WaF and 99 ± 6 % in WaW), after separating the biomass by centrifugation the COD(s + c) of the WaF and WaW was still at 1.4 g/L and 1.5 g/L, respectively. These values result from the sum of the non-degraded COD from the wastewaters and the soluble and colloidal COD that was present in the inoculum. So, after AD, the COD concentrations were still above the limit for discharge into the Portuguese public network.

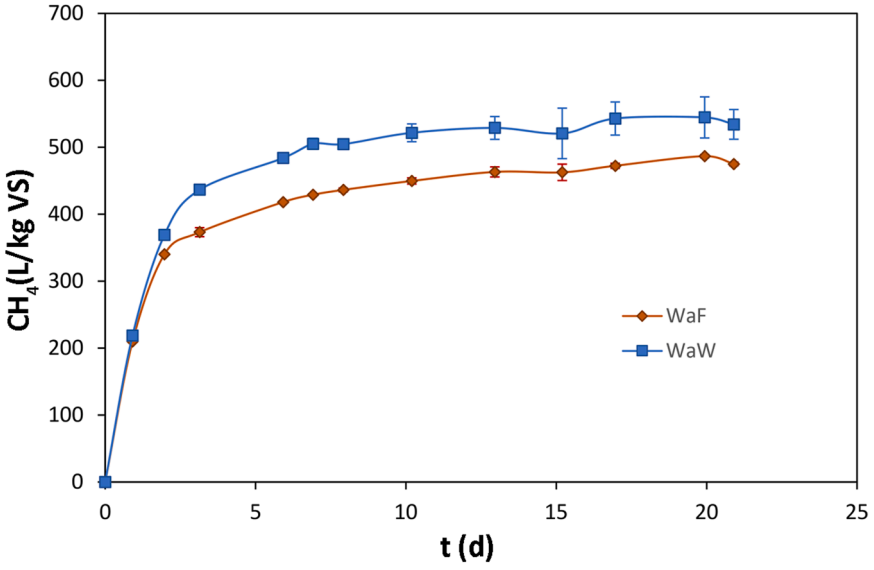

Fig. 4. Cumulative methane production over time, obtained from the anaerobic digestion of WaF and WaW streams, expressed as litres of CH_4 per kilogram of VS of wastewater added (L/kg VS); methane production by the inoculum in the blank assays was subtracted.

Using the EBH and CSL alternative culture medium we obtained high BNC yields, but also higher methane yields than those reported in the literature for similar wastewaters. For example, in a previous work, da Silva et al. [25] produced BNC from a culture medium containing by-products from different industries as alternative sources of sugars and protein, namely sugarcane molasses and corn steep liquor, and used anaerobic digestion to reduce the organic load of the final fermentation and washing wastewaters. The obtained methane yields in the batch assays were 387 ± 14 L/kg VS and 354 ± 4 L/kg VS for the WaF and WaW fractions, respectively, with COD removals of approximately 89 %; treatment of WaW in a continuous upflow anaerobic sludge blanket reactor reached 202 L/kg VS and COD removal around 60 %. Other authors later reported BNC production using glycerol, vinasse or whey as carbon sources, obtaining a maximum BNC production of 3.66 g/L after 28 days of cultivation in a 40 g/L glycerol medium. The effluent of this process was diluted to 2 g/L of COD and subjected to anaerobic digestion in sequencing batch bioreactors, reaching 77 % COD removal efficiency and a methane production of 3.63 mol/kg COD (81.3 L/kg COD) [35]. These reports show that BNC production can be achieved more sustainably by utilizing agro-industrial wastes, while adding value to the obtained effluents by producing biogas. In this work, we took this strategy one step further, by assessing the possibility of a sequential aerobic treatment or recycling of the wastewater, towards a more realistic and economically feasible industrial scenario.

3.2.3. Aerobic treatment of WaW after anaerobic digestion

The application of a subsequent aerobic treatment step, after the anaerobic stage, was tested using the WaW wastewater, as it better represents the total effluent generated in the BNC washing process; on the other hand, the results of the anaerobic digestion (Table 6) did not highlight a significant advantage in treating the WaF separately. Table 7 shows the characterization of WaW after anaerobic digestion and centrifugation to remove the biomass – WaW_AD. Table 8 shows the evolution of the CODt in the batch aerobic treatment assays.

Over a period of 96 h, the aerobic treatment was unsuccessful in further reducing the COD levels of the WaW_AD (Table 8). At the end of the assay, the pH of the WaW_AD wastewater shifted towards a more alkaline value (reaching a pH of approximately 9); thus, assays with added phosphate buffer (pH 7.0) were also done, to evaluate whether the higher pH had affected the aerobic treatment. Other controls were made in parallel, with acetate (an easily digestible substrate) and with diluted (1:2) WaW_AD (Table 8).

In CTR D, the COD removal was fast and acetate consumption was almost complete after 17 h, confirming the good activity of the aerobic sludge. A similar decrease in COD was observed for CTR C, which most probably corresponded to the acetate removal (approximately 750 mg/L of COD; the final COD value was similar to the COD of CTR A). Comparing CTR B with CTR C, it may be concluded that adjusting the pH to 7.0 with phosphate buffer had no positive effect on the aerobic degradability of the substrate. Dilution of WaW_AD also did not improve its degradability, as observed in CTR A, B and C.

These results suggest that the remaining compounds in WaW_AD are probably more difficult to metabolize, but do not seem to be inhibitory to the aerobic microbial community, since activity was observed when acetate was added. Similar results were obtained with wastewaters from BCN production with other culture media, namely a defined medium HS [36] and other alternative medium containing molasses and CSL [16], with no degradation of the organic fraction being detected in the aerobic treatment stage (data not shown). The inoculum used for the aerobic treatment consisted of an activated sludge from a municipal wastewater treatment plant. This sludge was not adapted to substrates such as EBH or CSL. However, exploratory tests with an inoculum collected from a different aerobic wastewater treatment plant that had processed molasses, thus having a microbial community adapted to a substrate rich in sugars and phenolic compounds, was also ineffective in digesting the WaW_AD (data not shown). This unexpected result deserves further assessment, as it is rather surprising to find out that a simple synthetic medium such as HS was not processed by the wastewater aerobic sludge. Nevertheless, these digested wastewaters contain water and nutrients and can be considered as another industrial waste worth valuing. Envisioning a truly integrated BNC production unit with minimal losses, the possibility of recycling the wastewaters after anaerobic digestion was assessed.

Table 7
Characterization of the WaW wastewater after anaerobic digestion and removal of the anaerobic sludge (WaW_AD).

	WaW_AD
pH	7.8
COD(s + c) (g/L)	1.5 ± 0.0 (Table 6)
CODs (g/L)	1.2 ± 0.0
TN (g/L)	1.2 ± 0.0
NH_4^+-N (g/L)	1.1 ± 0.1
NH_4^+ (g/L)	1.5 ± 0.1
PO_4^{3-}-P (mg/L)	5.4 ± 0.0

Table 8
Total chemical oxygen demand (CODt) of the WaW_AD and respective controls (CTR) during aerobic treatment.

	CODt (mg/L)		
Sample	0 h	17 h	96 h
WaW_AD	960	946	936
CTR A: WaW_AD 1:2, pH 7	775	424	n.d.
CTR B: WaW_AD 1:2, pH 7 + 0.5 g/L Acetate	1168	796	n.d.
CTR C: WaW_AD 1:2 + 0.5 g/L Acetate	1172	416	n.d.
CTR D: 0.5 g/L Acetate	776	66	n.d.

n.d. – not determined.

3.2.4. Recycling treated wastewater in BNC production

A large-scale BNC production unit requires high volumes of water (for example, the production of 1 ton/day (dry weight) requires around 800 m^3/day [11]). Thus, the wastewater treatment and its recycling into the BNC production process may represent an important cost reduction. Therefore, the effect of using recycled water (corresponding to 28 and 45 % of the culture medium volume) on the BNC yield was evaluated. Higher incorporation of recycled water was not possible in this study due to the low concentration of sugars in EBH and to the CSL (both in liquid form), which together represent around 50 % of the culture medium, on a volume basis. From Fig. 5, regardless of the type and amount of wastewater, the BNC yield (expressed as a percentage relative to the control) was not significantly affected ($p < 0.05$). In addition, different effluents from a municipal wastewater treatment plant (wastewaters collected before aerobic treatment (0.628 g/L of COD) and after aerobic treatment (0.072 of g/L COD) were tested in similar BNC production fermentations, producing the same results as the respective controls (data not shown). Altogether, these results demonstrate that recycled water can be used in the BNC production process. Although the corresponding wastewater (WaW), which gathers the several washing streams, as well as WaF, present values that are still well above the legal limits referenced in the Portuguese legal discard limit (Anex XVIII from the decree-law 236/98, 1st of August and under Regulation No. 169/2015, published in Diário da República, 2nd series — No. 71 — April 13, 2015), and it is not possible to dispose of the waters obtained after anaerobic treatment (WaW_AD and WaF_AD) into the public network, they can be reintegrated/recycled into the BNC production process without loss of the original BNC productivity.

4. Conclusions

This work demonstrated that eucalyptus bark hydrolysate (EBH) can be used as a cost-effective carbon source for bacterial nanocellulose

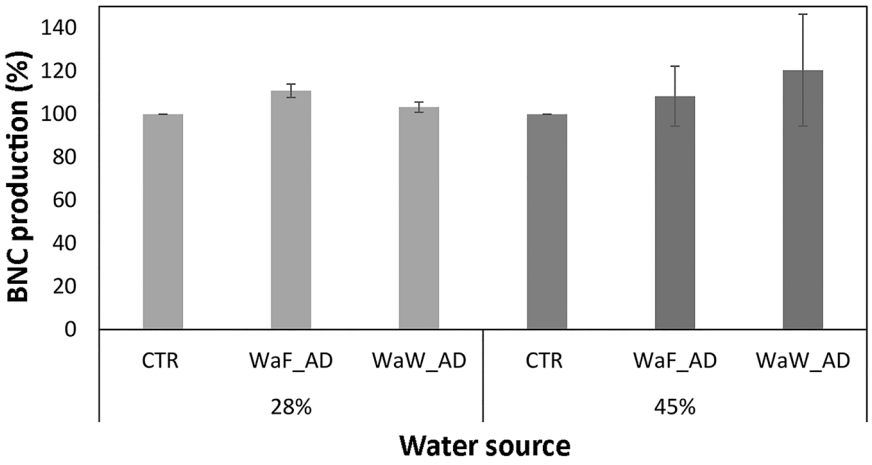

Fig. 5. Production of BNC from different wastewater sources, where CTR- distilled water; WaF_AD and WaW_AD- fractions of wastewater after anaerobic digestion. Data are presented as mean ± standard deviation of the experiments. One-way ANOVA and Bonferroni's Multiple Comparison Test indicated that there were no statistical differences in the BNC yield among the different assays.

(BNC) production, and its combination with other alternative nutrients (like CSL) leads to even more significant yields. Using RSM-CCD, the optimal medium composition was determined to be (% m/v) EBH 5.0 (total sugar), CSL 2.5 (total protein) and ammonium sulphate 0.3, producing a high BNC yield of 8.29 ± 0.21 g/L, a 39.7-fold increase over standard medium.

Furthermore, this work addressed the important aspects of the treatment and recycling of the wastewater generated in the BNC production. Wastewater streams after fermentation (WaF) and after BNC washing (WaW) revealed high organic loads, reaching COD values as high as 159 g/L and VS of 99.5 g/L. Anaerobic digestion (AD) of the wastewaters was highly efficient, achieving high methane yields of up to 544 L/kg VS, and COD reduction to a maximum of 1.5 g/L. The produced biogas can be converted into energy. However, a subsequent aerobic treatment of the anaerobically digested wastewater, intended to further reduce the organic load, was unsuccessful.

Although the COD after anaerobic digestion remained above the defined limit for discharge into the public wastewater network, it was successfully demonstrated that the anaerobically treated wastewater could be recycled within the BNC production process without negatively affecting the BNC yield. Adding 45 % of the treated wastewater to the culture medium had no effect on the BNC production yield compared with the optimal condition with distilled water. This finding has significant implications for water recycling in large-scale BNC production, potentially reducing the freshwater requirements and associated costs.

In sum, this work demonstrated the feasibility of an integrated approach for the optimization of BNC production from lignocellulosic wastes and the valorisation of the generated wastewaters through biogas production and water recycling. The strategies here developed can contribute to the economic and environmental sustainability of large-scale BNC manufacturing processes. Future work will address the assessment of multiple recycling tests on strain stability, BNC yield and properties, a more complete treatment of the wastewater and a higher incorporation of recycled water in the culture medium.

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CRediT authorship contribution statement

Ana Cristina Rodrigues: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Daniela Martins:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Miguel Gama:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Fernando Dourado:** Writing – review & editing, Supervision, Methodology, Conceptualization. **M. Salomé Duarte:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Ricardo Silva-Carvalho:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Susana Marques:** Writing – review & editing, Resources, Conceptualization. **Ana Júlia Cavaleiro:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.115442.

Data availability

Data will be made available on request.

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