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Optimization and characterization of bacterial nanocellulose produced by *Komagataeibacter rhaeticus* K3

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ABSTRACT

In this work, a novel Bacterial NanoCellulose (BNC) producing strain, from Kombucha tea, was isolated and characterized. Based on 16S rRNA analysis the strain was identified as *Komagataeibacter rhaeticus*. Under static culture, *K. rhaeticus* K3 produces membranes with a relaxed structure, as observed by Scanning Electron Microscopy (SEM). The addition of 2% (v/v) ethanol to the culture media enhanced by more than 3-fold of the BNC yield.

Response surface methodology (RSM) was performed with *K. rhaeticus K3*, using a new low cost Eucalyptus Biomass Hydrolysate (EBH). The maximum experimental BNC yield was of 5.46 g/L, obtained with the following composition: 31.4 g/L of EBH; 2.89% (v/v) of ethanol and 10.8 g/L of Yeast extract/peptone.

Texture Profile Analysis (TPA) of BNC membranes obtained using Hestrin-Schramm culture (HS) medium and optimized medium from EBH showed that membranes from EBH had higher resistance to compression, higher cohesiveness and resilience.

1. Introduction

Bacterial Nanocellulose (BNC) is a form of cellulose naturally synthesized by several species of *Acetobacteraceae*. Among the several BNC synthesizing bacteria, the most potent and most extensively studied are the *Komagataeibacter xylinus* and *Komagataeibacter hansenii* (Gama, Dourado & Bielecki, 2016). Due to its high purity, high porosity, high crystallinity, high degree of polymerization, high water holding capacity, low density, biocompatibility, non-toxicity and biodegradability, this biopolymer shows enormous economic potential in medical and composites applications, cosmetic and food industries and electronics (Campano, Balea, Blanco & Negro, 2016).

Despite the unique properties and interesting application potential, there are still strong limitations associated with its industrial production due to the high operational costs and low BNC yields. Extensive research has been done to promote higher productivities/yield by using low-cost raw materials such as rotten fruits, milk whey, carob and haricot bean and corn steep liquor as nitrogen source, molasse as carbon source, waste beer yeast, beverage industrial waste, fruit peels, rice bark and fruit juices as food industrial waste (Bilgi, Bayir, Sendemir-Urkmez & Hames, 2016; Campano et al., 2016; Fan et al., 2016; Jozala et al., 2016, Jozala et al., 2015). The use of wastewaters from textiles, pulp and paper and agricultural has also been reported (Campano et al., 2016; Chen et al., 2017; Cheng, Yang, Liu, Liu & Chen, 2017; Jahan, Kumar & Saxena, 2018; Jozala et al., 2016; Yan et al., 2012).

In parallel to the use of low-cost substrates, several researchers have been isolating, identifying and improving high-cellulose producing strains, including *K. xylinus* BPR 2002, *K. xylinus* BPR 2003, *K. xylinus* AS6, *K. xylinus* KJ-1, *K. hansenii* P2A, *K. swingsii* DSTGL01T, *K. rhaeticus* DSTGL02T, *K. rhaeticus* P1463, *K. oxydans, K. oboediens* and *K. persimmonis*, which display different characteristics in terms of bacteria motility, BNC morphology and yield (up to 9.49 g/L in the case of *K. rhaeticus* P1463, using apple juice as substrate) (Campano et al., 2016; Semjonovs et al., 2017). Therefore, the discovery of new strains may represent unique opportunity towards achieving high BNC yield and novel morphological and mechanical properties that may better suit different applications in cosmetics, food and biomedicine.

In this work, *Komagataeibacter rhaeticus* K3, isolated from Kombucha tea, was studied and compared with *Komagataeibacter hansenii* ATCC 53582 and *Komagataeibacter xylinus* E25. Also, the BNC production under static culture with the isolated strain was optimized using a low-cost substrate, a eucalyptus wood enzymatic hydrolysate, provided by RAIZ - Forest and Paper Research Institute (Portugal). The morphology and

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textural properties of the BNC produced by all of the above-mentioned strains was compared.

2. Materials and methods

2.1. Isolation, medium and growth conditions

This strain was isolated from inoculums from Kombucha tea. The tea was prepared using 5 g of green tea, 50 g of glucose and 500 mL of water. Fifty mL of Kombucha starter culture was added to the tea. The culture was incubated at room temperature for 10 days. One hundred mL of the suspension was transferred into 0.9 mL of 0.85% NaCl (Chempur, Poland). Serial dilutions from 10° to 10^{-10} were prepared using sterilized saline solution. An aliquot of 0.1 mL of each dilution was plated on Petri dishes with solid Hestrin-Schramm culture medium (HS) (Schramm & Hestrin, 1954) and incubated at 30 °C for 4 days. Single colonies were then picked and grown statically in liquid HS medium at 30 °C for 4 days. The novel strain was then identified (as described below) and used for cellulose production and characterization. The microorganism *Komagataeibacter rhaeticus* was labelled as K3 and it was deposited in Institute of Agricultural and Food Biotechnology (IAFB), Collection of Industrial Microbial Cultures, Warsaw, Poland with the registration number 2955.

2.2. Identification of Komagataeibacter rhaeticus

The new strain was identified by performing gram staining and colony morphology. The genomic DNA of *Komagataeibacter rhaeticus* was isolated according to the manufacturer's protocol (Gene MATRIX Bacterial and Yeast Genomic DNA Purification Kit and GeneMatrix Basic DNA Purification Kit, EURx, Poland), sequenced (unpublished data) and identified by using 16S rRNA sequence. A phylogenetic tree based on 16S rDNA gene sequences of type strains of the species of the family *Komagataeibacter* was constructed with the MEGA 7.0 (Kumar, Stecher & Tamura, 2016). Neighbour-joining tree was constructed with 35 sequences.

2.3. Bacterial cell culture conditions and growth media

For comparison purposes, *K. xylinus* E25 (a private strain from Bowil Biotech Ltd., Władysławowo, Poland) and *K. hansenii* ATCC 53582 (purchased from American Type Culture Collection) were also used, the former being representative of an immotile strain and the later a motile one (Florea, Reeve, Abbott, Freemont & Ellis, 2016; Jacek et al., 2019; Krystynowicz et al., 2002; Ryngajłło, Jacek, Cielecka, Kalinowska & Bielecki, 2019). All strains were cultivated in Hestrin–Schramm (HS) medium at 30 °C (Schramm & Hestrin, 1954), either in the liquid form (for BNC production under static culture), or in solid culture (for colony growth), with 2.0% (w/v) agar (Difco, USA). The HS medium contains (per liter): 20.0 g glucose (POCH, Poland), 5.0 g yeast extract (BTL, Poland), 5.0 g bacterial peptone (BTL, Poland), 2.7 g sodium phosphate dibasic (Chempur, Poland), 1.15 citric acid (Chempur, Poland) and 0.5 g magnesium sulphate (Chempur, Poland). The pH was adjusted to 5.7 with 80% (v/v) acetic acid (Chempur, Poland) before sterilization (which was done at 121 °C for 20 min).

2.4. Bacterial swarming motility assay

Pre-cultures of *K. xylinus*, *K. hansenii* and *K. rhaeticus* strains were diluted with 0.85% NaCl to reach an optical density of 0.1 at 600 nm. Next, 2 μL portions from each equilibrated culture were inoculated onto five HS plates containing 0.6% agar and 2% (v/v) cellulase (to prevent cellulose accumulation). After 4 days of incubation at 30 °C, the diameter of the colonies was measured using *Makroaufmassprogram* software (https://ruedig.de/tmp/messprogramm.htm).

2.5. Scanning electron microscopy (SEM) of BNC

For structural analysis, BNC membranes obtained after 7 days static culture of *K. xylinus* , *K. hansenii* and *K. rhaeticus* strains in HS medium, as described in 2.6, were purified by washing with 4% SDS (at 70 °C) (Sigma-Aldrich, Denmark) solution, 0.1% NaOH (Chempur, Poland) and whashed with distilled water until neutral pH (Fang, Kawano, Tajima & Kondo, 2015). The membranes were freeze-dried in a Christ Alpha model 1–4 LSC plus (Christ, Germany) and coated with gold. A scanning electron microscope FEI QUANTA 250 FEG (Thermo Fisher Scientific, MA, USA), operating at 2 kV, was used for the observation of three biological replicates from each strain at magnifications of 5000×, 20,000× and 40,000× . Representative micrographs were taken in triplicates for each magnification. The diameters of the cellulose fibers and pores were determined with the *Makroaufmassprogram* software (https://ruedig.de/tmp/messprogramm.htm), from 50 different sites on each of the SEM micrographs.

2.6. Effect of ethanol on BNC yield

For each strain, a single bacterial colony grown on HS agar seed medium was transferred to 5 mL of liquid HS medium and incubated at 30 °C for 4 days. Five% of the inoculum culture was added to a 250 mL Erlenmeyer flask containing 50 mL of HS medium supplemented with either 1% or 2% ethanol. The cultures were then incubated at 30 °C for 7 days. After incubation, the BNC membranes were picked and soaked in 2% NaOH solution overnight, and next in 1.5% acetic acid solution for 4 h. Afterwards, the membranes were washed with distilled water until neutral pH was reached. Then the membranes were dried at 55 °C in an oven to constant weight. The BNC yield was quantified, using Eq. (1) (see Section 2.9). For each strain, a control without ethanol was used as a reference.

2.7. Inoculum preparation and static culture fermentation

For the response surface methodology (RSM) assays (further detailed in 2.8), *K. rhaeticus* inoculum was grown in 1 L conical flasks, containing 100 mL of HS medium, for 48 h at 30 °C. Then, the formed cellulose pellicle was shaken to release the bacteria entrapped within the cellulose matrix into the residual media, which were used for further inoculations at 10% (v/v) of the final fermentation volume. The inoculated fresh media were incubated at 30 °C for 15 days (at a fixed culture medium depth of 2.5 cm height in 250 mL beakers). For the optimization of the composition of the culture medium, Eucalyptus Biomass Hydrolysate medium (EBH) was used, a sugar-rich liquor obtained by enzymatic hydrolysis of eucalyptus wood (RAIZ - Forest and Paper Research Institute, Portugal) (see conditions in Section 2.8). The medium thus contains the same components as the HS medium except for glucose, being replaced by EBH (EBH medium). As controls for RSM, BNC membranes were produced under the same conditions as above, but with HS (standard medium) and EBH media, both having 20.0 g/L of carbon (glucose equivalent) (Table 2).

2.8. Optimization of BNC fermentation by K. rhaeticus K3 using central composite design - Response Surface methodology (CCD-RSM)

RSM consists of a group of mathematical and statistical techniques used in the development of an adequate relationship between a response and a number of a known associated parameters (Khuri & Mukhopadhyay, 2010). A statistically designed study was conducted to investigate the individual and interactive effect of three medium components on the BNC yield: Glucose on EBH hydrolysate, yeast extract/peptone (YE/P; 50% of each) and ethanol. For this purpose, design expert 7.1.5 (Stat-Ease. Inc. USA. Windows operating system) was used for the experimental design and CCD-RSM statistical analysis of EBH medium with *K. rhaeticus*. Tables 1 and 2 show the experimental design used for the

Table 1
Level of factors chosen for the experimental central composite design response surface methodology for EBH medium.

Factor	Name	Low actual	High actual	Low coded (−1)	High coded (+1)	Mean	Std. Dev
A	Carbon (g/L)	12.0	35	4.2	42.8	23.5	9.8
B	Ethanol% (v/v)	1.0	3.5	0.2	4.4	2.3	1.1
C	Nitrogen (g/L)	5.0	15.0	1.6	18.4	10.0	5.5

Table 2
BNC yield results EBH medium for CCD-RSM.

Run	Carbon: Glucose equivalent (EBH) (g/L)		Ethanol (%v/v)	YE/P (g/L)	BNC yield (g/L)
1	12.0	1.0		5.0	3.62 ± 0.06
2	**23.5**	**4.4**		**10.0**	**5.57 ± 0.04**
3	**23.5**	**2.25**		**10.0**	**5.44 ± 0.307**
4	23.5	0.15		10.0	3.93 ± 0.10
5	12	1.0		5.0	3.69 ± 0.067
6	23.5	2.25		1.6	3.09 ± 0.23
7	4.2	2.25		10.0	1.88 ± 0.059
8	**35**	**3.5**		**5.0**	**5.21 ± 0.50**
9	12	3.5		15.0	4.25 ± 0.14
10	**23.5**	**2.25**		**10.0**	**5.34 ± 0.35**
11	35	1.0		15.0	4.73 ± 0.15
12	**35**	**3.5**		**5.0**	**5.14 ± 0.41**
13	**23.5**	**2.25**		**18.4**	**5.28 ± 0.34**
14	**23.5**	**2.25**		**10.0**	**5.63 ± 0.29**
15	12	3.5		15.0	4.20 ± 0.11
16	**42.8**	**3.5**		**5.0**	**5.56 ± 0.06**
17	**23.5**	**2.25**		**10.0**	**5.81 ± 0.29**
18	**23.5**	**2.25**		**10.0**	**5.85 ± 0.19**
19	35.0	1.00		15.0	4.87 ± 0.50
20	**40.0**	**2.00**		**10.0**	**5.44± 0.19**
21	**40.0**	**2.00**		**20.0**	**5.70 ± 0.13**
22	**40.0**	**2.00**		**30.0**	**5.19 ± 0.06**
23	40.0	4.00		10.0	4.85 ± 0.06
24	40.0	4.00		20.0	4.96 ± 0.06
25	40.0	4.00		30.0	4.74 ± 0.06
Control EBH	**20.0**	**2.00**		**10.0**	**5.27 ± 0.12**
Control HS	20.0	2.00		10.0	3.44 ± 0.04

optimization. All the runs in table 2 were tested in triplicate and all combinations of the fermentation medium included 3.39 g/L of Na_2HPO_4 and 1.26 g/L of citric acid. The initial pH used was set to 5.5 in all media. The model was evaluated using the Fisher´s statistical test for analysis or variance (ANOVA). Finally, three-dimensional curves of the response surfaces were generated using the same statistical approach. All the runs were made in triplicates and the values of the BNC yield represent the average and standard deviation of the values retrieved. The average and standard deviations were calculated with GraphPad prism 7. The results from the CCD experiments are shown in table 2.

2.9. Bacterial nanocellulose yield and water retention capacity

After 15 days of fermentation, the BNC produced was washed with NaOH 0.1 M at room temperature. Afterwards, the membranes were washed with distilled water, also at room temperature, until the pH became that of the distilled water. The washed membranes were then weighed before and after drying to constant mass at 37 °C, to calculate the volumetric yield of BNC (g/L) and water retention capacity (WHC), according to the following equations:

$$\text{BNC (g/L)} = \frac{dried\ BNC\ (g)}{culture\ medium\ volume(L)} \quad (1)$$

$$\text{WHC (g water removed/g of dried BNC)} = \frac{wet\ BNC\ (g) - dried\ BNC\ (g)}{dried\ BNC\ (g)} \quad (2)$$

WHC measurements were done in triplicates. All values represent the average and standard deviation of the values retrieved.

2.10. Textural profile analysis

Texture profile analysis (TPA) was performed using a Texture analyser HD plus C. For comparison, BNC membranes from *K. hansenii*, *K. xylinum* and *K. rhaeticus*, were produced with HS medium (as described in Section 2.7). Ethanol (2% v/v) was used only for *K. xylinum* and *K. rhaeticus*. For each BNC membrane, a 7.5 cm diameter compression plate (P/75) was used and compression tests were done under the following specifications: 50% of strain (of original height); a crosshead pre-test of 1 mm/s, a test speed of 0.5 mm/s, a post-test speed of 0.5 mm/s and a trigger force of 5 g. These analyses were processed with Exponent Stable micros Systems (Windows), which allowed to assess the hardness (g), springiness (%), cohesiveness (%) and resilience (%) of the BNC membranes. Further explanation on how was determined each parameter, can be found in supplemental material (Fig. S2.). The measurements were made in sextuplicates. All values represent the average and standard deviation of the values retrieved.

2.11. Analytical methods

2.11.1. Total sugar and protein quantification

High Performance Liquid Chromatography (HPLC) with the Aminex HPX-87H IEX column, PU-2080 Plus pump (JASCO), DG-2080-53 degasser (JASCO), AS2057-Plus automatic sample injector (JASCO) and a 2031 Plus RI detector (JASCO) was used to identify and quantify the concentrations of cellobiose, glucose and xylose in EBH hydrolysate and the initial and residual sugars in culture medium. The following conditions were used: mobile phase (5 mM H_2SO_4) flow rate at 0.05 ml/min; column temperature 35 °C. The injected volume was 20 μL. Cellobiose,

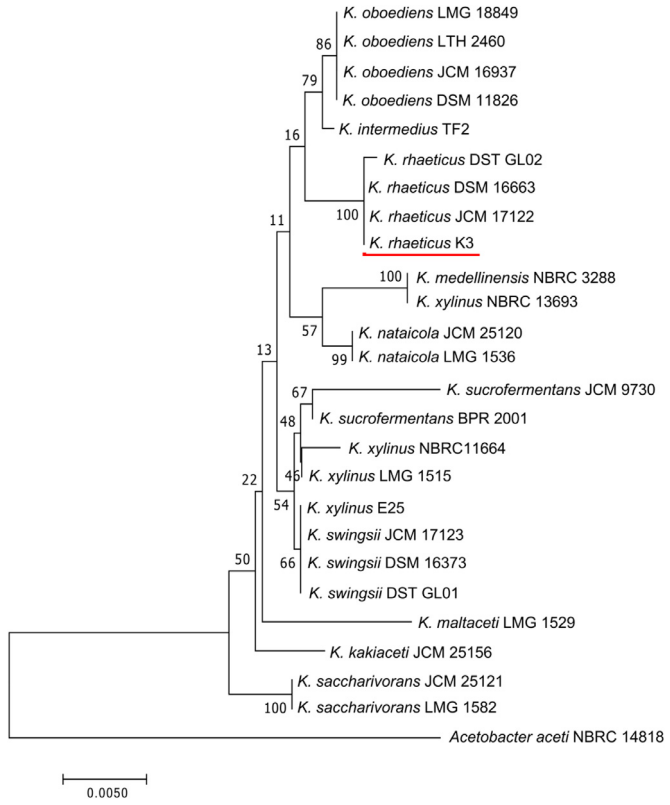

Fig. 1. Phylogenetic tree based on the sequence of 16S rRNA genes from bacterial cellulose producers using the neighbour-joining method.

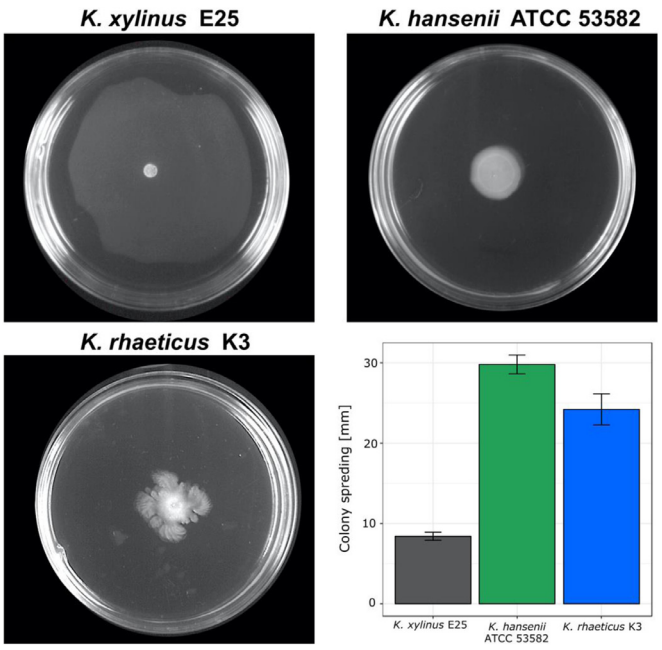

Fig. 2. Swarming plate assay conducted for *K. xylinus* E25, *K. hansenii* ATCC 53582 and *K. rhaeticus* K3. The images were taken on the 4th day of incubation.

glucose and xylose concentrations were determined based on calibration curves obtained using the pure compounds with concentrations ranging from 0.01 g/L to 25 g/L. The obtained sugar composition in EBH hydrolysate was glucose 131 g/L, xylose 13.2 g/L and cellobiose 3.70 g/L.

3. Results and discussion

3.1. Phylogenetic analysis and motility assays of the isolated strain

A BLAST search of the GenBank database using 1486 bp 16 S rRNA gene sequence of strain K3 showed its similarity with many members of the genus *Komagataeibacter*. A phylogenetic tree (Fig. 1) based on 16S rRNA gene sequences of members of the genus *Komagataeibacter* was constructed according to the bootstrap test of neighbour-joining algorithm method of Saitou and Nei (1987) with MEGA7 (Kumar et al., 2016). This tree shows the close phylogenetic association of strain K3 with certain other *Komagataeibacter* species. Phylogenetic analysis indicated that the strain K3 consistently falls into a clade together with *Komagataeibacter rhaeticus* strains DSM 16663, JCM 17122, DST GLO2 with a similarity of 100% (Fig. 1).

To describe the motility of *K. rhaeticus, K. xylinus* - an immotile strain - and *K. hansenii* - a motile strain - were used as controls. Soft-agar motility assay was performed to analyse the swarming motility of bacteria strains (Ha, Kuchma & O'Toole, 2014). Briefly, density-equilibrated cultures of examined strains were spotted on a semi-solid agar medium. The spreading of the 'colonies' was measured every day in millimetres (mm).

We observed that *K. rhaeticus* is motile, as the diameters of *K. hansenii* and *K. rhaeticus* spots on agar plates begun to spread on the third day of incubation. For *K. rhaeticus*, the mean diameter of the colony after 4 days was of 24 mm, whereas for *K. hansenii* it was of 30 mm (Fig. 2). Regarding the dimensions of the bacteria, *K. hansenii*, *K. xylinus* and *K. rhaeticus* cells had lengths of ~ 5.2 μm, ~ 3.9 μm and ~ 2.0 μm, respectively (Fig S1).

3.2. Characterization of BNC pellicle produced by K. rhaeticus K3

The structure of BNC membranes synthesized by all studied strains was compared by SEM analysis on both the upper and bottom surfaces. Also, of the pore size and thickness of the fibres were measured. These results (Fig. 3) show that when all strains were cultured in HS medium, the BNC membrane from *K. rhaeticus*, has much higher porosity and thicker fibres than the ones from the other strains. Wild-type strains from the genus *Komagataeibacter* have been observed to produce membranes with a compact and densely packed structure without visible pores in the upper part of the membrane (Vazquez, Foresti, Cerrutti & Galvagno, 2013). However, the morphology of the BNC membranes is known to change according to the composition of the culture medium (Al-Shamary & Al-Darwash, 2013; Molina-Ramírez et al., 2017).

3.3. Effect of ethanol on BNC yield

From a literature review, ethanol was observed to increase the BNC production yield. Certain *Komagataeibacter* strains are known to use ethanol as an additional carbon source. Supplementing a culture medium with ethanol allowed also to repressing the spontaneous mutations of BNC, while increasing cells´ ATP production (Krystynowicz et al., 2002; Naritomi, Kouda, Yano & Yoshinaga, 1998; Park, Jung & Park, 2003). To evaluate the impact of ethanol on the BNC yield, static culture assays were done with all strains, in HS medium supplemented with either 1% or 2% ethanol (Section 2.6). The produced BNC membranes from *K. rhaeticus* and *K. xylinus* showed an irregular top surface. However, adding ethanol to the culture medium of *K. rhaeticus*, allowed the formation of membranes with a smoother top surface (Fig 4b). Both *K. rhaeticus* and *K. xylinus* produced more cellulose with ethanol supplementation. The addition of 2% ethanol caused more than a 3-fold increase in the BNC production by *K. rhaeticus* (Fig. 4b), reaching 5.55 g/L of dry mass after 7 days. The addition of ethanol to the *K. hansenii* culture, did not affect the BNC yield. As for *K. xylinus*, the addition of 2% ethanol allowed for 7.5 times increased in the BNC yield.

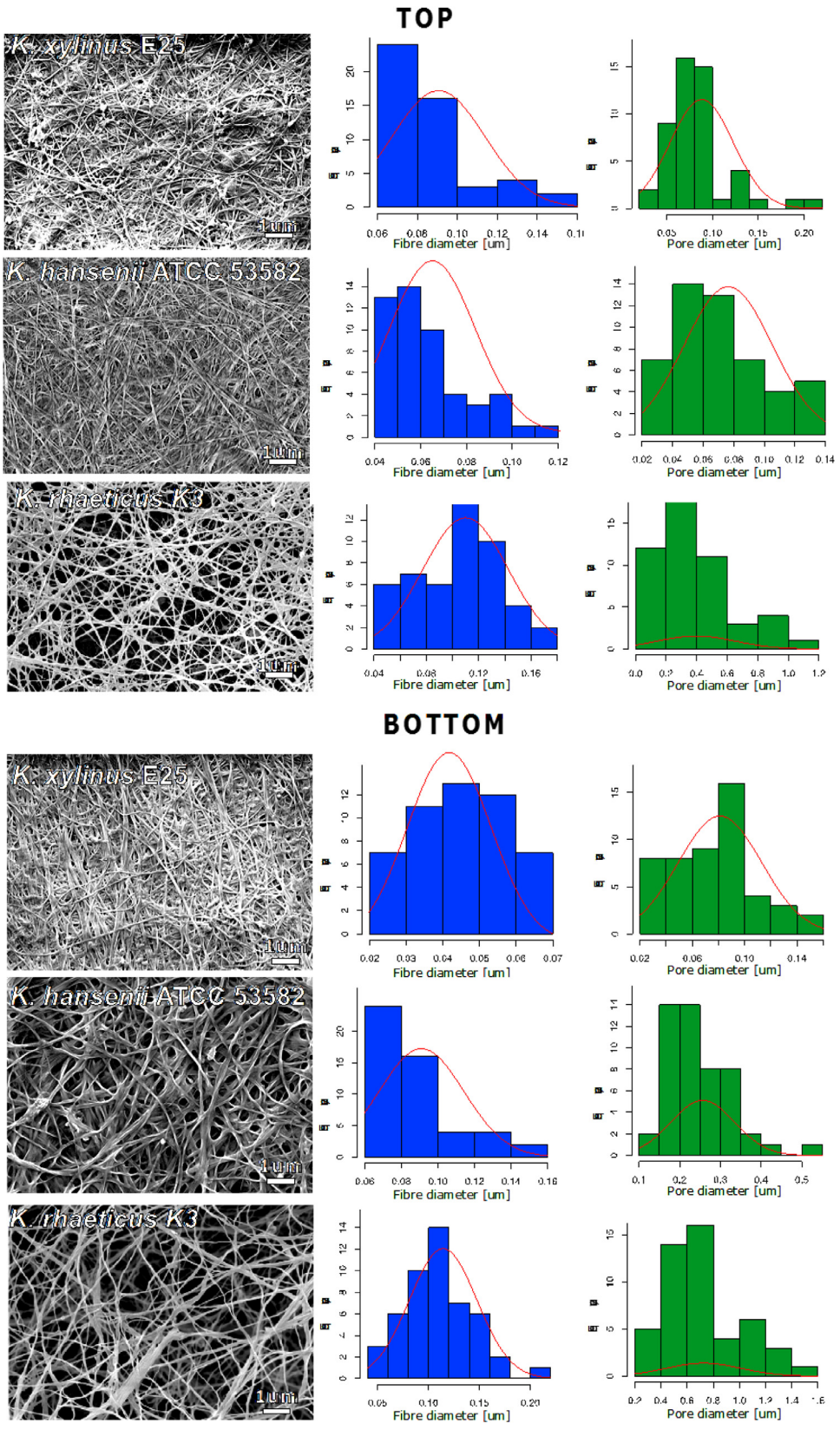

Fig. 3. Characteristics of BNC membranes. Left column- scanning electron micrographs of top and bottom sides of membranes produced by *K. xylinus* E25, *K. hansenii* ATCC 53582 and *K. rhaeticus* K3 strains after 7 days static culture in Hestrin-Schramm medium. Middle column- fibre diameter. Right column- pore diameter. Analyses were done using Scanning Electron Microscope FEI, Quanta FEG 250. All views with ETD detector under 40,000× magnification.

According to Basu et al. (Basu, Vadanan & Lim, 2018), there is a correlation between motility and the efficiency of BNC production, the more motile strains producing more cellulose. While not excluding the synergistic impact of nutrient sources, our results also confirm these observations, since, as observed above (Fig. 2), with HS medium (without ethanol), both *K. rhaeticus* and *K. hansenii*, being mobile strains, exhibit higher BNC yield than *K. xylinus* (Fig. 4b). The same pattern was also observed by using HS media with 1% v/v and 2% v/v ethanol, where higher BNC yields were observed with motile strains (*K. rhaeticus* and *K. hansenii*) (Fig. 4b).

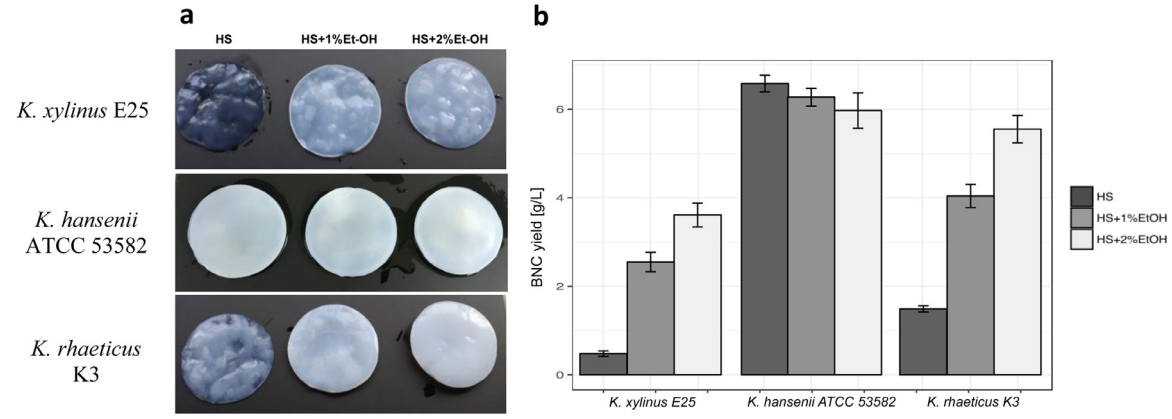

Fig. 4. Effect of ethanol on BNC production by *K. xylinus* E25, *K. hansenii* ATCC 53582 and *K. rhaeticus* K3: (a) purified BNC membranes after 7 days culture in HS medium, (b) comparison of cellulose production efficiency in HS medium with addition of 1% and 2% ethanol after 7 days.

Table 3
ANOVA analysis of the model obtained for EBH.

Source	Sum of squares	Df	Mean square	*F*value	*p*-value Prob > *F*	
Model	**8.69**	**9**	**0.97**	**12.20**	**<0.0001**	*Significant*
A-carbon	**3.93**	**1**	**3.93**	**49.63**	**<0.0001**	*Significant*
B-ethanol	**1.62**	**1**	**1.62**	**20.51**	**0.0006**	*Significant*
C-nitrogen	0.019	1	0.019	0.24	0.6324	
AB	0.089	1	0.089	1.12	0.3084	
AC	0.095	1	0.095	1.20	0.2925	
BC	1.559E−004	1	1.559E−004	1.970E−003	0.9653	
A^2	**2.27**	**1**	**2.27**	**28.68**	**0.0001**	*Significant*
B^2	**2.34**	**1**	**2.34**	**29.52**	**0.0001**	Significant
C^2	**0.37**	**1**	**0.37**	**4.68**	**0.0498**	Significant
Lack of Fit	**0.87**	**10**	**0.087**	**1.61**	**0.3824**	
R^2				**0.8942**		
Adj R^2				0.8209		
Pred R^2				0.6366		
Adeq. Precision				**11.225**		

3.4. Optimization of culture medium with K. rhaeticus *K3 by response surface methodology-central composite design*

CCD-RSM has been widely used with the goal of maximizing BNC yield (Bilgi et al., 2016; Ha et al., 2014; Naritomi et al., 1998; Park et al., 2003; Mohammadkazemi, Azin & Ashori, 2015). A graphical and numerical optimization was made to achieve the highest BNC production yield using EBH with *K. rhaeticus* (see experimental design; Table 2).

Before CCD-RSM, an analysis of the BNC yield from each run was performed. The highest values were obtained with runs 2, 8, 12, 13, 16, 20, 21, 22 and the control EBH ($p < 0.05$) (Table 2). No significant differences in the values of the BNC yield were observed between the mentioned runs ($p > 0.5$), resulting in an overall average yield of 5.46 g/L BNC. In all these cases, EBH medium was used at glucose, nitrogen and ethanol concentrations higher than, respectively, 20 g/L,10 g/L and 2% (v/v). Regarding the control assays, a lower BNC yield ($p < 0.5$) was obtained using HS medium (3.44 ± 0.04 g/L), as compared to EBH control. The micro-nutrients that putatively exist in EBH enhanced the BNC production, as compared to the HS medium. Similar observations were observed in the literature for other alternative substrates (Bilgi et al., 2016; Rodrigues et al., 2019; Silva et al., 2019).

$$\text{Equation 1}: \text{BNC\_yield\_EBH} = -0.0962 + 2.25 * \text{Carbon} + 1.55 * \text{ethanol} + 0.524 * \text{Nitrogen} - 0.0585 * \text{Carbon} * \text{ethanol} + 0.113 * \text{Carbon} * \text{nitrogen} - 3.84\text{E}-003 * \text{ethanol} * \text{nitrogen} - 0.368\, Carbon^2 - 0.256 * ethanol^2 - 0.269 * nitrogen^2$$

{Degree of freedom = 9; *F*-value = 12.20; *p*-value <0.0001; $R^2 = 0.8942$}

Table 3 displays the ANOVA analysis of the model built by CCD-RSM for BNC yield optimization of *K. rhaeticus*. In addition, the significance of each parameter (CS, NS and ethanol) and the interaction between them were assessed. Analysis of variance (ANOVA) statistical analysis was carried out followed by Fisher's Least Significant Difference (LSD) test. Regarding the RSM analysis of BNC yield with *K. rhaeticus* with EBH medium, a second polynomial order model was obtained (Eq. (1)). According to Table 3, the *F*-value of 12.20 and of *p*-value Prob > *F* (<0.0001) imply that this model is adequate (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" (test for comparing lack-of-fit variance with pure error variance) of 1.61 and the *p*-value Prob > *F* of 0.3824 indicates that the Lack of Fit is non-significant (>0.05) relative to the pure error, i.e. there is 38.24% chance that a "Lack of Fit F-value" could occur due to noise. For the controlled terms, *"A-Carbon", "B-ethanol", "A^2-Carbon^2"* and *"B^2-ethanol^2"* these were all significant (*p*-value Prob > *F* < 0.05, Table 3). Higher R^2 values indicate better correlation between the predicted and experimental data. It was suggested that the R^2 value should be at least of 0.80, for a good model fitness (Joglekar & May 1987). The obtained R^2 was of 0.8942, which indicates that 10.58% of the total variation could not be explained by the empirical model (Table 3). 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 (11.225). This is higher than 4, indicating that this model can be used to navigate the design space. Therefore, the individual and interactive effect of model terms on BNC production can be interpreted by the 3D graphs of CCD provided in Fig. 5, which represents a saddle-like curve for the studied parameters.

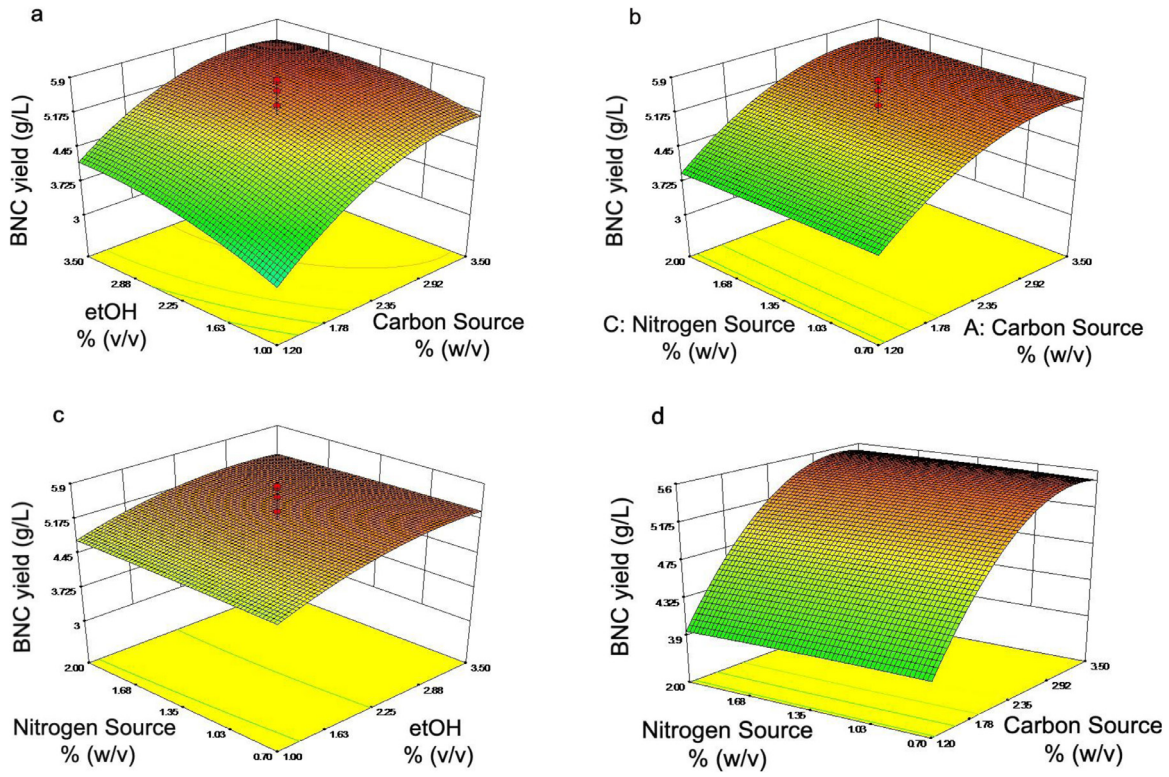

Fig. 5. 3D graphs of *K. rhaeticus* RSM optimization with EBH culture medium; surface curve for BNC yield (a) as a function of carbon and ethanol. (b) as a function of carbon and nitrogen. (c) as a function of nitrogen and ethanol and (d) optimization graph as a function of carbon and ethanol.

Table 4
Predicted and experimental values of BNC yield with EBH medium for *K. rhaeticus* K3 strain, under optimized culture conditions.

Assay	Carbon (g/L)	Et-OH% (v/v)	Nitrogen (g/L)	Predicted BNC yield (g/L)	Experimental BNC yield (g/L)	95%CL low	95%CL high
1	**23.5**	**2.25**	**13.5**	**5.59**	**5.36 +0.22**	**5.36**	**5.89**
2	**31.4**	**2.89**	**10.8**	**5.80**	**5.44+0.55**	**5.56**	**6.04**
3	23.5	2.25	10.0	5.57	5.36+0.88	5.32	5.83
4	35.0	2.25	20.0	5.76	6.55+0.90	5.51	6.02

After numerical optimization, several solutions were found with the highest yields. EBH concentration of 23.5 g/L or higher provided the highest BNC yield (Fig. 5). However, the increase of EBH, which increases glucose concentration, may lead to a higher production of gluconic acid, known to decrease the medium's pH, thus inhibiting BNC production (Tsouko et al., 2015). The same effect was perceived with ethanol, where the highest BNC yield was obtained with ethanol, up to a concentration of 3% (v/v) (Fig. 5). BNC yield increased by 45% with optimized ethanol supplementation and by 211% with optimized EBH addition (Fig. 5 and Table 3) An opposite effect has been reported with *K. xylinus* ATCC 700178 (Rodrigues et al., 2019; Silva et al., 2019). The more promising medium compositions were used in additional fermentation assays (Table 4 and Fig. 5). ANOVA analysis with the experimental results showed that all experimental results after RSM optimization were similar ($p > 0.20$). Also, ANOVA analysis of experimental and predicted values, showed a lack of significance in all EBH medium tested ($p > 0.20$). Therefore, the second polynomial model was validated.

3.5. Texture profile analysis of BNC membranes

Texture analysis of the membrane was also performed with the goal of understanding the differences between strains (*K. rhaeticus* vs *K. hansenii* vs *K. xylinus*) and between the culture media used (*K. rhaeticus* HS vs *K. rhaeticus* EBH). Due to the lack of significance within the four assays from Table 4 and these being representative of the best conditions to maximize BNC production (Table 4), assays 1 and 2 were chosen for further analysis (Table 4). For comparison, BNC membranes were produced by *K. rhaeticus*, *K. xylinus* and *K. hansenii* with HS medium (see experimental section of TPA). All BNC membranes produced were analysed through TPA and WHC measurements.

The parameter "hardness" measures the degree of stiffness of the BNC membrane (maximum force in the first compression). BNC from *K. rhaeticus* EBH_Assay1, showed the highest hardness value ($p < 0.05$). The lowest hardness values were found with *K. rhaeticus* HS ($p < 0.05$). Springiness describes how well the BNC membrane springs back after the first deformation occurred. The highest value was obtained with BNC from *K. hansenii* ($p < 0.001$), the BNC membranes produced by other strains showing a much lower and similar springiness values ($p > 0.4$) (Fig. 6). Cohesiveness is defined by how well the BNC membrane withstands a second compression relative to its resistance under the first deformation. Membranes produced by *K. hansenii* and *K. rhaeticus* cultured in EBH medium presented similar ($p > 0.10$) cohesiveness, although the values were relatively low (30%) (Fig. 6). Lower values of cohesiveness were found on membranes produced by *K. xylinus* and *K. rhaeticus* HS ($p < 0.004$) (Fig. 6). Moreover, *K. xylinus* and *K. rhaeticus* HS membranes presented similar cohesiveness ($p > 0.70$) (Fig. 6). The resilience measures the ability to regain the original height after 5 s of the first compression. All membranes showed very low resilience (Fig. 6). It is known that BNC membranes can reswell to some extent after compression, but this is a very slow process. Overall, all membranes assessed in Fig. 6 present low cohesiveness and resilience. The more relevant

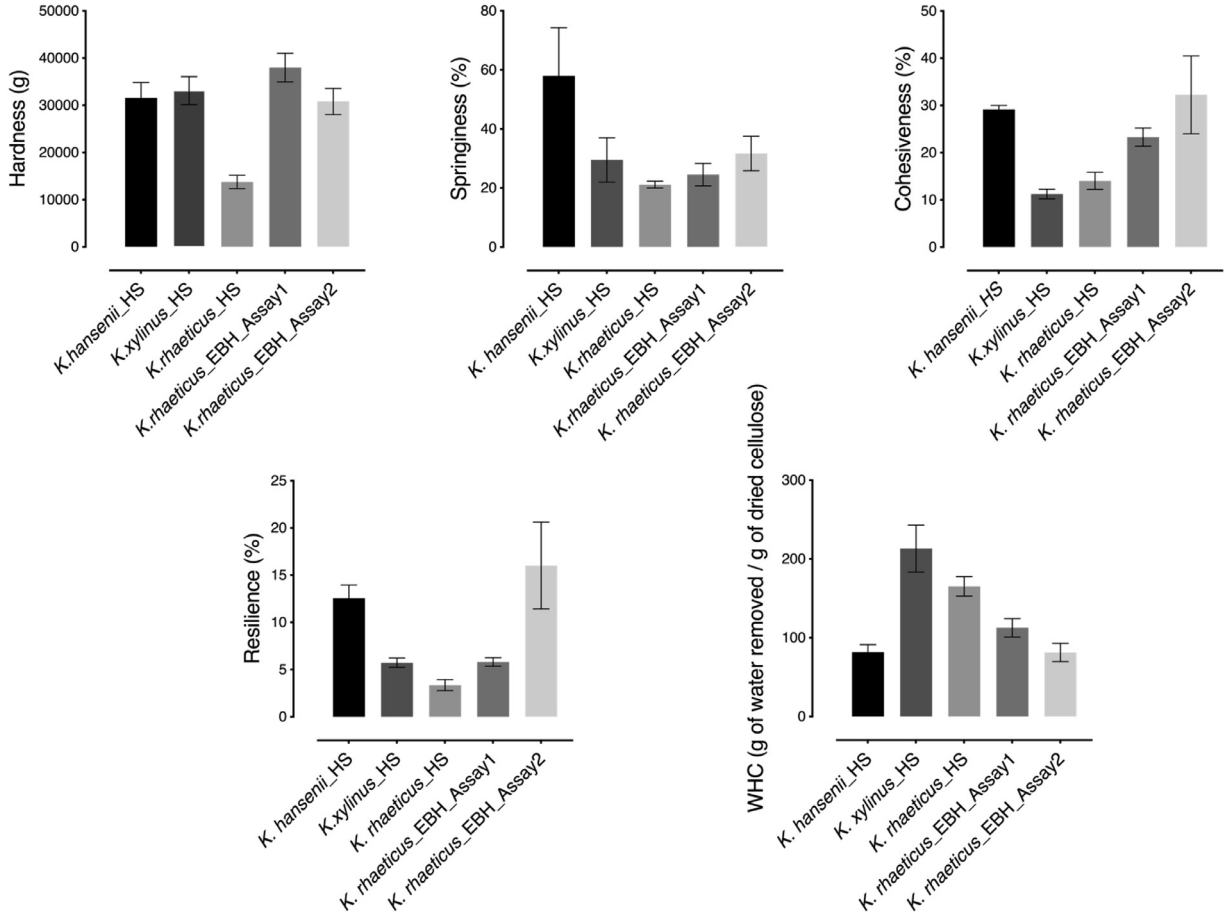

Fig. 6. Hardness, springiness, cohesiveness, resilience, firmness and WHC analysis of BNC membranes produced by *K. rhaeticus* in different culture medium and by *K. hansenii* and by *K. xylinus* in HS medium.

differences observed in the textural properties concern the lower hardness of *K. rhaeticus* HS, the higher springiness of *K. hansenii* and the differences (in hardness, cohesiveness and resilience) between BNC membranes produced with different culture medium (*K. rhaeticus* HS vs *K. rhaeticus* EBH) (Fig. 6). More specifically, membranes produced with EBH medium displayed higher hardness, cohesiveness and resilience than membranes produced with HS medium. The use of different culture media can indeed interfere on the BNC membrane production during static fermentation. Similar behaviour was encountered in Khumbar's et al. (Kumbhar, Rajwade & Paknikar, 2015) work, as the use of a low cost substrates formulation (pineapple and watermelon peel) led to higher hardness, cohesiveness and resilience, in comparison to HS medium. Membranes from *K. hansenii* and *K. rhaeticus* with EBH medium displayed similar WHC (Fig. 6), despite being cultured under different medium culture compositions. Chung and Shyu (Chung & Shyu, 1999) showed that BNC membranes with lower WHC had higher resistance to compression (higher hardness). Indeed, a straightforward rational relating the textural properties (in particular the more relevant differences pointed out above) could not be found. Thus, we suggest that structural features of the membranes (porosity, network crosslinking, interconnections between the several BNC layers) may justify the noted differences. Both the culture medium (*K. rhaeticus* HS vs *K. rhaeticus* EBH) and the strain used (*K. hansenii* vs *K. rhaeticus* vs *K. xylinus*) may lead to significant differences. This is a relevant feature concerning the exploitation of BNC in the development of fruit mimetics (Dourado et al., 2016). Although the well-known Nata de Coco is used for human consumption for many years, there is relatively little exploitation of BNC in the food industry (Klemm et al., 2018). This product may be further developed by using different additives, such as soluble polysaccharides, in order to tune the textural properties, mimetizing for instance different kinds of fruit. This will be further developed in future work.

4. Conclusion

In this work, a novel bacterial cellulose (BNC) producing strain, *K. rhaeticus* K3, isolated from Kombucha tea was characterized by SEM and 16S rRNA analysis. The effect of ethanol on the BNC production yield under static culture was studied. Also, a CCD-RSM analysis of BNC production with *K. rhaeticus* and EBH medium was performed. The highest average BNC yield was 5.46 g/L. In terms of textural properties, the use of EBH medium allowed to produce membranes with higher resistance to compression, higher cohesiveness and resilience. On the other hand, the use of HS medium allowed to produce BNC with higher water retention capacity (WHC). Therefore, it is possible to produce BNC membranes with different textural characteristics with *K. rhaeticus,* depending on the desired application.

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.

Author contributions

FD, SB and MG conceived and designed research. PJ and FGS conducted experiments. FGS, PJ and FD analysed data. FGS and PJ wrote the manuscript. All authors read and approved the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2020.100022.

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