



## Development and characterization of bacterial cellulose produced by cashew tree residues as alternative carbon source



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### ABSTRACT

Bacterial cellulose (BC) has been extensively exploited for applications in materials science, biomedical and technological fields. The BC production demands culture media rich in carbon sources. Agro-forestry residues constitute an interesting source of nutrients for microorganism, but they are frequently wasted. For cashew crop, exudate is periodically extracted from the tree trunks to increase the production of cashew nut, the most valuable product from cashew trees that produces about 700 g of exudate/year, which remains wasted. Here, we associated the nutritional properties of residues from cashew tree with the need of carbon sources for BC, in attempt to valorize the residue and to decrease the costs of BC production. The carbon source from Hestrin Schramm culture medium was totally or partially replaced by cashew tree residues and the BC production was evaluated. The produced BC membrane in static medium was characterized by FTIR, SEM and TGA and the kinetics of production was determined, suggesting the cashew tree residues as a potential carbon source for BC production.

### 1. Introduction

The use of cellulose in paper making is one of its most noble and known applications (Klemm et al., 2005). The exceptional physical chemical properties, availability and renewability of cellulose have been widening its ranges of applications in the last decades. Encouraged by the decrease in paper demand, cellulose has been used to develop new materials and composites, including the multifunctional and/or nanostructured ones (Carreira et al., 2011; Joye and McClements 2016; Wang et al., 2016).

Cellulose is a homopolymer of D-glucopyranose residues linked by  $\beta$ -(1  $\rightarrow$  4) glycosidic linkages (Esa et al., 2014), synthesized by plants and other organisms, such as bacteria, fungi and animals (Trovatti 2013; Garcia et al., 2016). The most representative producers within the bacteria kingdom belong to *Gluconacetobacter*, *Acetobacter* and *Koma-*

*gataebacter* genera (De Salvi et al., 2014; Shoda and Sugano 2005; Machado et al., 2016). The cellulose produced by these microorganisms, known as bacterial cellulose (BC) or biocellulose, is obtained as a gel-like three-dimensional mat formed by entangled nanofibrils of cellulose. BC is produced in high pure state and it has unique mechanical properties related to the intrinsic properties of cellulose macromolecules, associated to the morphology of the membranes woven by the bacteria, which represents a crucial point for its application in the development of composites, in which the nanofibers are used for reinforcement (Tabarsa et al., 2017). Despite the promising properties of BC, its application remains limited because of the high costs of production, including the culture media. In attempt to overcome this disadvantage, some strategies have been proposed, such as the genetic improvement of the cellulose producing microorganisms and the formulation of low cost culture media (Cacicedo et al., 2016).

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The conventional nutrient source for BC production in academic studies is Hestrin and Schramm (HS) culture medium, composed by glucose, yeast extract, peptone and mineral salts (Hestrin and Schramm 1954). Many modifications on the composition of HS medium have been proposed, such as the use of mono- and disaccharides like fructose, sucrose, maltose, cellobiose, xylose, galactose and alditols, which have been successfully used as carbon sources (Ishihara et al., 2002; Keshk and Sameshima 2005). Industrial wastes or by-products from agro-forest and food industry have been also used for BC production, for instance, Konjac powder (Hong and Qiu 2008), beet molasses (Keshk et al., 2006), sugar-cane molasses (Tyagi and Suresh 2016), corn steep liquor (Noro et al., 2004), as source of carbon, nitrogen and other nutrients.

Regarding the utilization of residues/by-products, Brazil is an essentially an agroforest country, in which cashew crop and processing represent an important branch of the food chain. The main products in cashew industry are the cashew nut and cashew juice, and the main by-products are cashew pulp and the exudate. Exudate is periodically extracted from the tree trunks to increase the production of cashew nut, the most valuable product from cashew trees. Each tree of the specie *Anacardium occidentale* produces about 700 g of exudate/year, which remain wasted (Costa et al., 1996). The cashew tree exudate (CTE) is rich in mono- and oligosaccharides, arabinogalactan-protein and mineral salts (Menestrina et al., 1998; Pereira-Netto et al., 2007; Rodrigues et al., 1993; Silva et al., 2010). CTE is constituted of ca. 70% of a branched heteropolysaccharide, the called cashew gum (CG). Its main polymeric chain is formed by D-galactopyranose units linked by  $\beta$ -(1  $\rightarrow$  4) glycosidic linkages (Rodrigues et al., 1993). Arabinose, rhamnose, glucose, and glucuronic acid are other sugars that can integrate the branched chemical structure of CG. The annual production of CTE and CG is at least 68.000 and 48.000 tons/year, respectively (Rodrigues et al., 1993). These large amounts of wasted by-products show the economic importance in adding value to these residues.

These residues have been extensively used in pharmaceutical industry (as excipient binder), in biotechnology industry and food industry (in beverages, as thickening agent, gelling agent and colloidal stabilizer) (Kumar et al., 2012). However, it is the first time that cashew crop residues are exploited as a source of nutrients for BC production.

Considering that BC has shown tremendous potential as an effective biopolymer in various fields, the use of CTE and CG as a source of nutrients for its production is a good opportunity to reuse the wastes, adding value to these by-products which can decrease the costs of BC production. Thus, the hypotheses of this research is to use CTE and CG as a source of nutrients for BC production. For such, the carbon source from HS medium was replaced (totally or partially) by CTE or CG. BC production was determined and BC was characterized to check if it kept its intrinsic properties.

## 2. Materials and methods

### 2.1. Materials

Anhydrous D-glucopyranose and ethanol, citric acid,  $\text{KH}_2\text{PO}_4$ ,  $\text{Na}_2\text{HPO}_4$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , all P.A. grade were purchased from Synth<sup>®</sup>. Bacteriologic peptone, agar and yeast extract were purchased from Merck. CTE was collected at Center of Agrarian Sciences – CCA at Federal University of Piauí (UFPI), Teresina, Piauí state, Brazil, and used as such, or after purification steps (gum polysaccharide, CG).

### 2.2. CTE purification – isolation of CG

CG isolation was carried out using the methodology described by Rodrigues et al., 1993, with some modifications. Shortly, an aqueous solution of CTE (10%, w/v) was prepared and stirred for 12 h. The solution was then neutralized with 1 M NaOH solution and filtered. CG was subsequently precipitated by adding ethanol (4  $\times$  in volume) to the

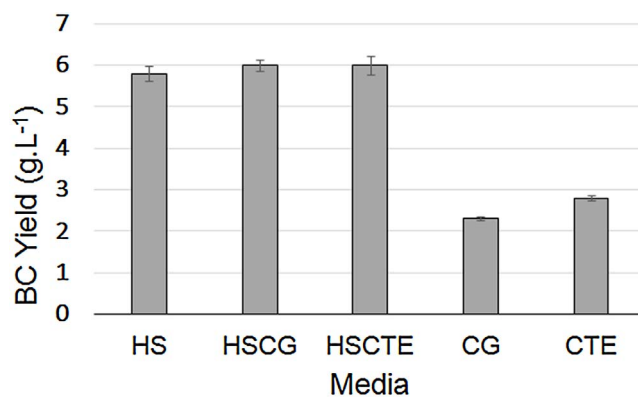


Fig. 1. BC production from HS, HSCG, HSCTE, CG and CTE at 7th days time point.

CTE aqueous solution. Precipitated CG was separated by filtration and successively washed with ethanol and acetone, generating a white powder, which was oven dried (30 °C) for 24 h and stored at room temperature.

### 2.3. Microorganism maintenance and pre-inoculum

*Komagataeibacter rhaeticus* previously isolated by Santos et al. (2014) was maintained in HS solid culture medium at 4–8 °C. One single colony from the solid medium was used to inoculated into 50 mL of modified HS liquid medium (50 g L<sup>-1</sup> glucose, 4 g L<sup>-1</sup> of yeast extract, 0.73 g L<sup>-1</sup> of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g L<sup>-1</sup>  $\text{KH}_2\text{PO}_4$ , 20 g L<sup>-1</sup> ethanol and distilled water, 1000 mL, pH 6.5) and incubated at 28 °C for 24 h in static condition. The liquid medium was then homogenized and used as the pre-inoculum.

### 2.4. BC production and culture media

The production of the BC membranes was carried out by cultivation of 5 mL from the previously prepared *K. rhaeticus* suspension in 45 mL of liquid culture media in a 250 mL Erlenmeyer flask, at 28 °C for 168 h (7 days), without agitation. HS was used as the standard culture medium. The media prepared using cashew residues as alternative carbon sources were formulated by replacing total or partial glucose by CTE or CG and labeled HSCTE (25 g L<sup>-1</sup> glucose plus 25 g L<sup>-1</sup> of CTE), HSCG (25 g L<sup>-1</sup> glucose plus 25 g L<sup>-1</sup> of CG), CG (50 g L<sup>-1</sup> of CG) and CTE (50 g L<sup>-1</sup> of CTE).

### 2.5. Production kinetics, purification and drying the BC membranes

The samples for BC production kinetics study were carried out at 0, 48, 72, 96, 120, 144 and 168 h time points using the best culture medium (HSCTE) based on BC production. It was not possible to evaluate the BC production at 24 h because only a thin and no homogeneous membrane was obtained. The BC membranes were exhaustively washed with distilled water in order to remove the impurities from the culture medium. Then, they were treated with an aqueous solution of 0.1 M NaOH at 80 °C for 45 min and washed with distilled water until neutral pH. The purified BC membranes were dried at 38 °C for 48 h and weighted.

### 2.6. Field emission gun scanning electron microscopy (FEG-SEM)

SEM experiments were carried out using samples previously coated with evaporated carbon. The images were obtained using the JEOL T-300 microscope operating at 2 kV.

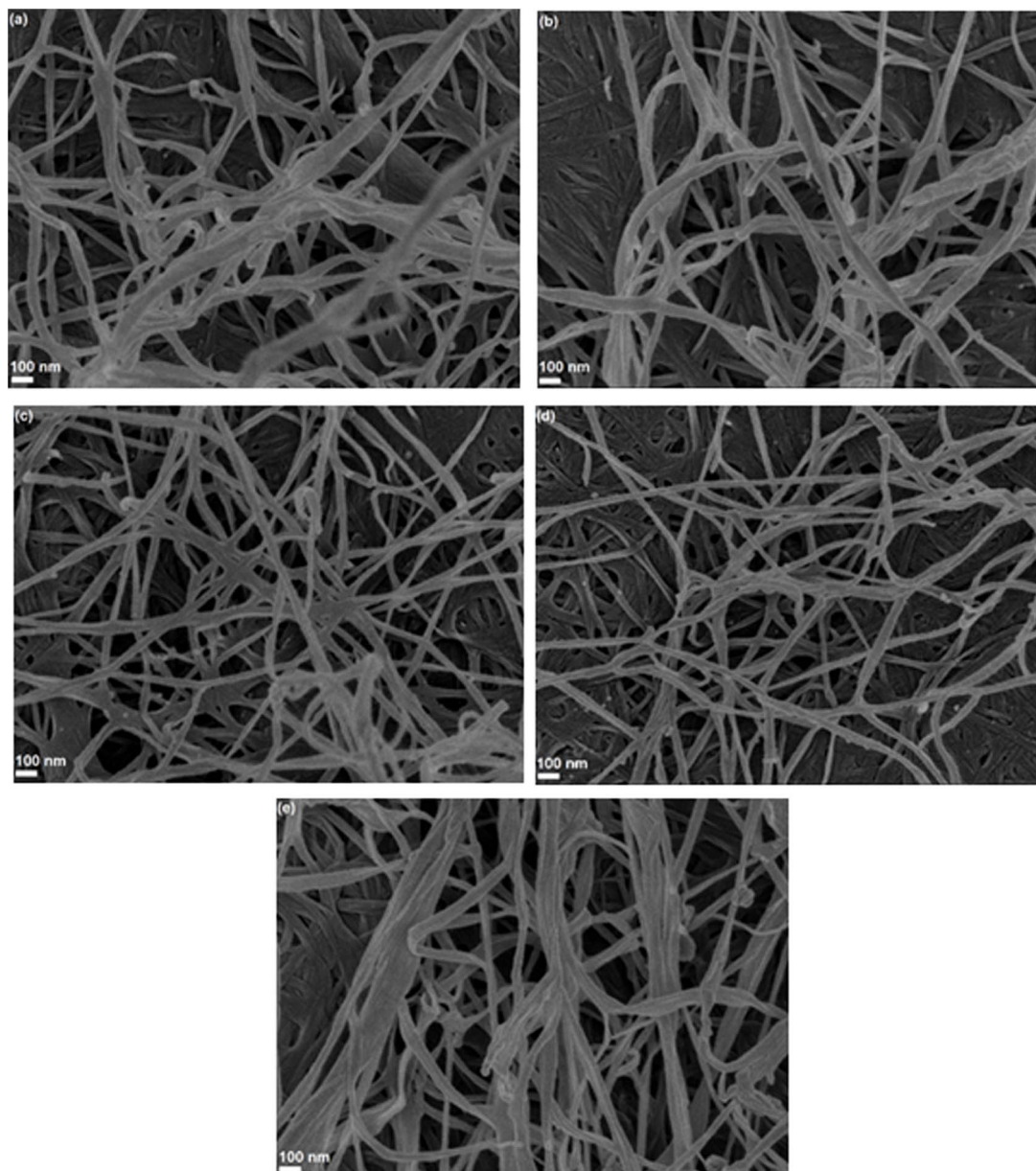


Fig. 2. SEM images of the BC samples obtained at 7 days of incubation in HS medium (a), HSCG (b), HSCTE (c), CG (d) and CTE (e). Scale bar = 100 nm.

### 2.7. FTIR spectroscopy

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (FTIR) spectra were acquired using a Perkin Elmer Spectrum 100 spectrometer equipped with a single horizontal Golden Gate ATR cell. The resolution was  $4\text{ cm}^{-1}$ , 16 scans. Spectra were collected from  $4000$  to  $600\text{ cm}^{-1}$ .

### 2.8. Thermogravimetric (TGA/DTG analysis)

Thermogravimetric analyses were performed using an SDT Q600 (TA Instruments, USA), at  $10\text{ }^\circ\text{C}/\text{min}$  under nitrogen atmosphere ( $30\text{ mL}/\text{min}$ ).

### 2.9. XRD patterns

XRD patterns of BC membranes produced at HS and the best culture medium (HSCTE) based on BC production were acquired using a Shimadzu XDR6000 diffractometer with  $\text{Cu-K}\alpha$  radiation

( $\lambda = 1.5406\text{ \AA}$ ),  $2\theta$  in the range between  $10^\circ$ – $80^\circ$  and a scan rate of  $2^\circ\text{ min}^{-1}$ . The crystallinity (C) of the BC samples was determined using the SEGAL method (Segal et al. 1959). The crystallinity index of the BC membranes was calculated according to the Segal method (Eq. (1)).

$$\text{CrI} = [(I_{002} - I_{\text{am}})/I_{002}] \times 100 \quad (1)$$

### 2.10. Thickness analysis

The thickness of BC membranes produced at HS and the best culture medium (HSCTE) based on BC production was accurately measured using a digital micrometer MDC-Lite (Mitutoyo®) in five random positions around the samples, in sextuplicate and average values were used in calculations.

### 2.11. Mechanical properties

Mechanical properties of membranes produced at HS and the best culture medium (HSCTE) based on BC production were evaluated using

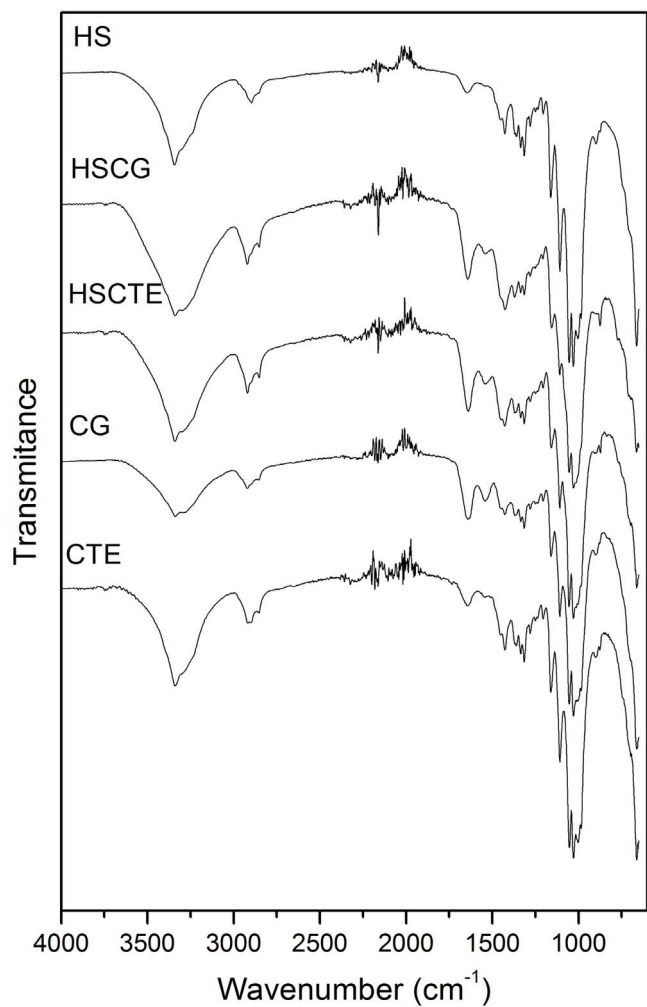


Fig. 3. FTIR spectra of the BC samples from HS, HSCG, HSCTE, CG and CTE medium at 7 days incubation.

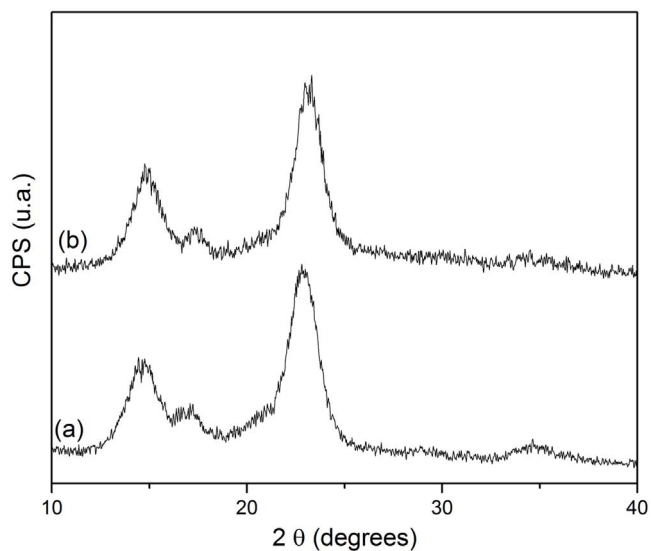


Fig. 5. XRD patterns of the BC samples. (a) BC sample obtained by HS Medium; (b) BC sample obtained by HSCTE medium after 7 days of incubation.

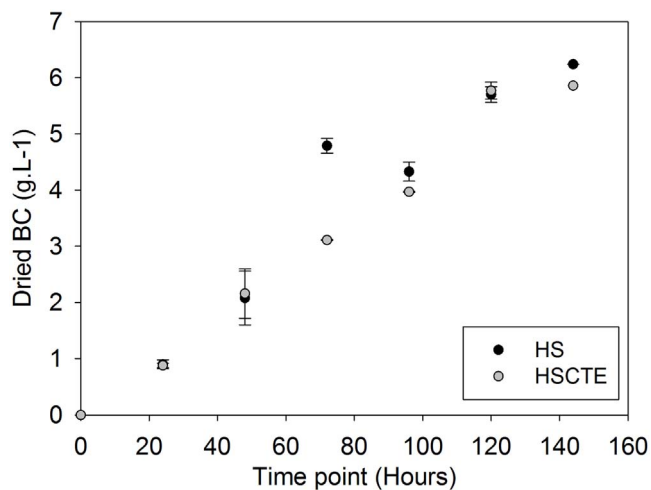


Fig. 6. Kinetics of BC production (g/L) from HS medium and HSCTE.

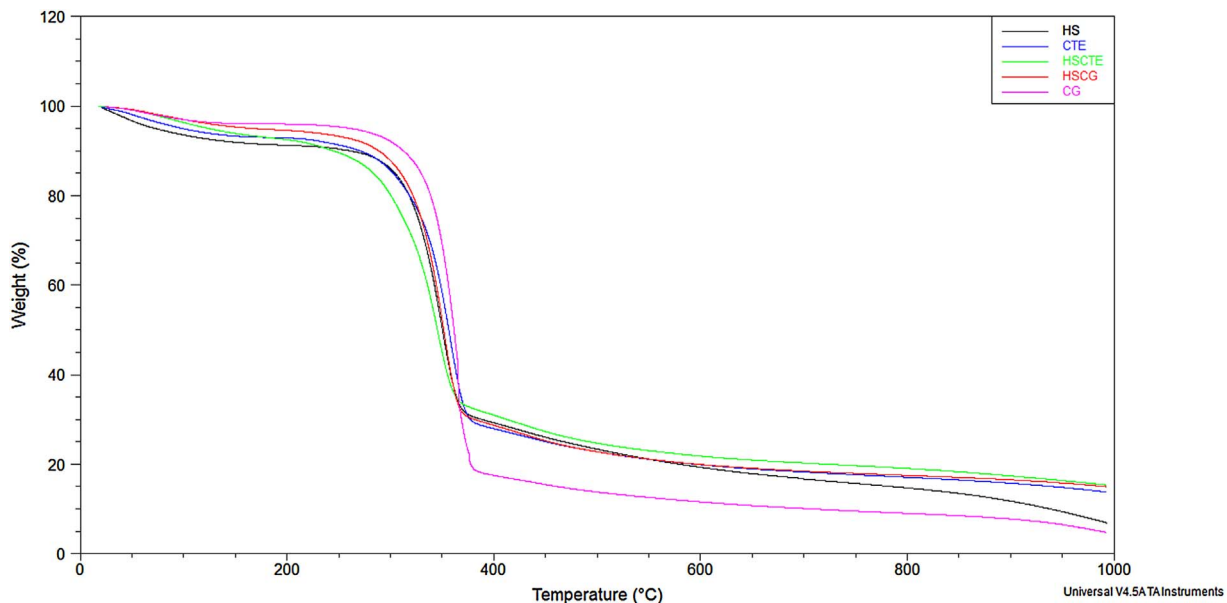


Fig. 4. TG curves of the BC samples from HS, HSCG, HSCTE, CG and CTE medium at 7 days incubation.

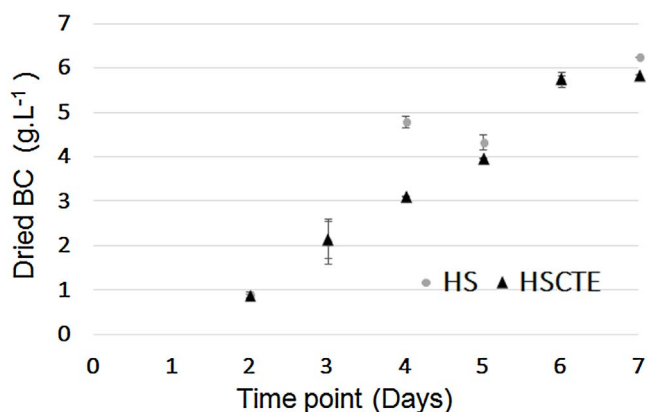


Fig. 7. Thickness of BC membranes as a function of time (2–7 days) and culture medium (reference HS and HSCTE supplemented medium) (media  $\pm$  standard deviation).

a texture analyzer TA-XT2 (Stable Micro Systems) equipped with a spherical puncturing probe (5 mm). BC sections were fixed on a holder with a circular hole ( $D = 10$  mm) and the probe was driven through the membranes with a speed of  $1 \text{ mm s}^{-1}$  and  $0.005 \text{ kg}$  trigger force. Force-displacement curves were recorded until the film rupture and used to determine the puncture strength ( $P_s$ ), elongation at break ( $E_b$ ), perforation energy ( $E_p$ ) and modulus at puncture, which were calculated from Eqs. (1)–(4) (Meneguín et al., 2014; Limmatvapirat et al., 2007):

$$P_s = F_{\max}/A_{CS} \quad (2)$$

where  $F_{\max}$  (N) is the maximum applied force and  $A_{CS}$  ( $\text{mm}^2$ ) the cross-sectional area of the membranes placed on circular hole, with  $A_{CS} = 2rh$ , where  $r$  is the hole radius and  $h$  is the membrane thickness.

$$E_b = [(r2 + d)0.5 - r]/r \cdot 100 \quad (3)$$

where  $r$  (mm) is the radius of the exposed membrane on the orifice plate and  $d$  is the probe displacement from de point of contact to point of puncture.

$$E_p = AUC/V \quad (4)$$

where  $AUC$  is the area under the curve force versus displacement and  $V$  the membrane volume ( $V = \pi r^2 h$ , where  $r$  is the hole radius and  $h$  is the membrane thickness) placed on the orifice plate.

$$\text{Modulus at puncture} = P_s/E_b \quad (4)$$

### 3. Results and discussion

#### 3.1. BC production

The effect of the total or partial replacement of carbon sources from the culture medium with CTE or CG on the BC production was investigated and the results ranged from  $2.3 \text{ g L}^{-1}$  (CG medium) to  $6.0 \text{ g L}^{-1}$  (HSCG and HSCTE media), as showed in Fig. 1. 2.3 and  $2.8 \text{ g L}^{-1}$  of BC were produced using pure CG and CTE residues, respectively. The media HSCTE and HSCG led to the yield about  $6 \text{ g L}^{-1}$ , essentially identical to the yield obtained using the reference HS medium, indicating that the substitution of 50% of glucose did not affect the BC production.

Recently, industrial waste or by-products have been proposed for

Table 1  
Mechanical properties of BC membranes cultivated in HS medium and HS-CTE medium.

Culture medium	$P_s$ (MPa)	$E_b$ (%)	$E_p$ ( $\times 10^{-4} \text{ kJ m}^{-3}$ )	Modulus at puncture (GPa)
HS	$268.12 \pm 35.46$	$32.91 \pm 7.22$	$16.41 \pm 6.23$	$8.02 \pm 2.16$
HSCTE	$144.86 \pm 44.90$	$39.69 \pm 8.74$	$5.16 \pm 1.48$	$4.04 \pm 1.81$

producing BC and in most cases, the yields are lower than those in HS medium, but in literature, generally, the production of BC using residues is variable (Mohammadkazemi et al., 2015). For instance, wastes from brewery industries led to a BC production from  $1.74$  to  $2.41 \text{ g L}^{-1}$  (Khattak et al., 2015); from wastewater rice wine distillery, BC production was about  $6.31 \text{ g L}^{-1}$  and from thin stillage it was about  $6.27 \text{ g L}^{-1}$  (Wu and Liu, 2012). Wastewater from lipids fermentation produced  $0.4$ – $0.6 \text{ g L}^{-1}$ , (Huang et al., 2016), and from molasses,  $1.64 \text{ g L}^{-1}$  (Çakar et al., 2014) and  $7.62 \text{ g L}^{-1}$  (Bae and Shoda 2004). The use of sugars, such as mannitol and sucrose were also used as alternative carbon sources for BC production, leading to  $1.9$  and  $1.6 \text{ g L}^{-1}$ , respectively (Mohammadkazemi et al., 2015). Sucrose and glucose led to  $2$  and  $3 \text{ g L}^{-1}$ , respectively (Castro et al., 2012).

The cashew residues as like the other residues abovementioned, are frequently wasted or sold for low values. According to the scientific literature, they can be exploited as an alternative nutrient source adding value to the final product. In order to confirm the significance of these residues to reduce production costs, which are mainly related to the culture medium, a brief economic viability is demonstrated. For this purpose, the cost of  $1 \text{ L}$  of culture medium was evaluated, totaling US\$ 18.90 for HS, US\$ 16.42 for HSCTE and HSCG and US\$ 12.68 for CTE and CG media, which means an important cost reduction ranging from 16.5 to 33.0%.

#### 3.2. Field emission gun scanning electron microscopy (FEG-SEM)

SEM images displayed in Fig. 2 show the surface morphology of the BC membranes produced from the different culture media. The analysis performed did not show significant differences among the nanofibers obtained from the different carbon sources. All the samples displayed heterogeneous 3-D network porous structure, in which the nanofibers of about  $20$ – $50 \text{ nm}$  diameter are randomly arranged.

#### 3.3. FTIR spectroscopy

FTIR spectroscopy of BC produced in HS medium, HSCG, HSCTE, CTE, CG was carried out in order to detect any effect of the composition of culture media on profile of cellulose spectrum. FTIR spectra of the samples (Fig. 3) were similar, indicating that the polymer had similar chemical structure. The typical bands of cellulose are observed at  $1102$  and  $1165 \text{ cm}^{-1}$  corresponding to C–O symmetric stretching of primary alcohol, and C–O–C antisymmetric bridge stretching, respectively, and at about  $3300 \text{ cm}^{-1}$  (broad band) corresponding to OH stretching. The band at  $1340 \text{ cm}^{-1}$  corresponding to the C–H deformation ( $\text{CH}_3$  or O–H in plane bending) and the band centered at  $1400 \text{ cm}^{-1}$  was related to  $\text{CH}_2$  bending and OH in plane bending. Other bands related to O–H bending of adsorbed water (at  $1645 \text{ cm}^{-1}$ ), CH stretching of  $\text{CH}_2$  and  $\text{CH}_3$  groups (at  $2900 \text{ cm}^{-1}$ ) can also be seen.

#### 3.4. Thermogravimetric (TGA analysis)

Fig. 4 shows TG curves. All samples showed a similar thermal behavior, the curves obtained for BC membranes displayed two mass losses. The first one, a small mass loss starting at room temperature up to  $180 \text{ }^\circ\text{C}$  related to loss of surface water ( $\sim 3.9$ – $9.10\%$ ) as it was also observed by De Salvi et al. (2012). The second mass loss event was attributed to the sample decomposition process at temperatures between  $200$  and  $400 \text{ }^\circ\text{C}$ , with maximum decomposition ( $T_{\text{onset}}$ ) at around

345–364 °C (71–82%). The degradation of cellulose, includes depolymerization, dehydration and decomposition of glucose units (Barud et al., 2008).

BC membranes produced from CG medium showed a higher thermal stability in comparison with others cultures mediums, verified by the higher  $T_{onset}$ . CG medium can be forming more effective chemical interactions (like hydrogen bonds) with hydroxyls group of bacterial cellulose and its increase thermal stability.

### 3.5. XRD patterns

XRD patterns of BC produced in HS and HSCTE medium are depicted in Fig. 5, and shows peaks at  $2\theta$  angles of 14.8°, 16.7° and 22° related to the (1 $\bar{1}$ 0), (110) and (200) planes, respectively. Broad diffraction peaks are observed at 22.76° and 22.80° for both samples. These peaks are assigned to the characteristic interplanar spacing of native cellulose type I (Kim et al., 2006).

Segal equation CrI expresses the relative degree of crystallinity,  $I_{002}$  is the maximum intensity (in arbitrary units) of the 002 lattice diffraction and  $I_{am}$  is the intensity of diffraction in the same units at  $2\theta$  angle 22.76°. CrI value was about 78% and about 80% for HS and HSCTE, respectively. These values are similar to others reported in the literature (Trovatti et al., 2011; Wu and Liu, 2013; Li et al., 2015).

### 3.6. BC production kinetics

The culture medium HSCTE was selected as the most promising for BC production, thus, it was used to compare with the kinetics of production in HS medium. Fig. 6 shows the BC production kinetics in both culture media followed similar profiles and results. The production rate ( $\text{g L}^{-1} \text{h}^{-1}$ ) of the membranes was  $0.041 \text{ g L}^{-1} \text{h}^{-1}$  from 24 to 96 h of cultivation with a slightly increase to  $0.048 \text{ g L}^{-1} \text{h}^{-1}$  at 120 h, followed by a decrease to 0.040 at 144 h of incubation. These data indicated the BC production was kept constant up to 96 h, because the culture media provided the suitable conditions (nutrients, low metabolites accumulation in the system) for maintenance of the cell growth and the subsequent BC production. At 120 h of incubation, the microorganisms reached the maximum production rate, followed, as expected, by a bacterial bath system, the decrease in the production rate, indicating the decline of the number of live cells, probably related to the exhaustion of the culture media by depletion of the nutrients and increase of the metabolites. However, deeper studies should be further developed to confirm this affirmation, for example, residual glucose test in all media.

The increase of BC membranes thickness from 0.014 mm on the second day to 0.019 mm on the seventh day, in function of time is shown in Fig. 7. BC thickness remained constant up to 3 days time point and then, trends to increase gradually over the days following the kinetics of the BC excretion by the microorganism.

### 3.7. Mechanical properties

Table 1 shows the mechanical properties ( $P_s$ ,  $E_b$ ,  $E_p$  and modulus at puncture) of BC membranes produced in both HS and HSCTE media.

The mechanical properties of the membranes from HS medium were higher than those obtained from HSCTE medium, however both samples showed high  $P_s$  and modulus at puncture. These results cannot be directly compared to the mechanical properties found in literature because the results here were obtained using the puncture mode, while in most works the results are obtained in tension mode. However, the high mechanical performance of BC remained interesting and the  $P_s$  values for both samples is at the same range of the tensile strength from literature obtained in tension (Barud et al., 2011; Perotti et al., 2011).

The method used here to determine the mechanical properties of BC membranes showed the  $E_b$  in perforation mode was quite different from that property determined in tension mode. In the last, the elongation at

break is normally lower than 5% (Rani et al., 2011; Khattak et al., 2015), while in perforation mode it was about 30–40%. These properties, described here for the first time, suggest the use of BC membranes for applications in which it will be exposed to deformation forces.

Mechanical results can be considered an important indicator of the uniform size of cellulose nanofibers and its well-organized state, besides being related to its high degree of entanglement by inter- and intramolecular hydrogen bonding, which frequently conduces to high Young's modulus (Abeer et al., 2014; Barud et al., 2015). Although the rigid polymeric network, cashew gum may contain natural plasticizers that intercalate them with cellulosic nanofibers, allowing the flexibility and reorientation when a force is applied, resulting in some difference in relation to the HS membranes. In addition, the cashew gum may increase the viscosity of culture medium, interfering with the growth of cellulose nanofibers. This hypothesis should be addressed to further studies.

Although the differences among the  $P_s$  values for the membranes produced in the HS and supplemented HSCTE medium, both substrates were efficiently used for BC production, suggesting cashew tree exudate medium as a promising alternative carbon source for BC production.

## 4. Conclusion

Despite important researches on the search for alternative media for BC production, the strategic alignment between reduction of costs and increase of productivity still remains a major challenge.

The results compiled in this work, in general, show that cashew tree exudate and cashew gum have great potential to be used as carbon source to produce BC from *K. rhaeticus* with an important cost reduction, which can reach 33%. Besides, it was possible to demonstrate that HSCTE and HSCG supplemented media allowed the same BC yield as HS medium. Importantly, morphology and the properties of BC produced by cashew tree derivative were in agreement with those obtained from the conventionally used culture media.

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