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ORIGINAL PAPER

Bacterial cellulose nanofiber-based films incorporating gelatin hydrolysate from tilapia skin: production, characterization and cytotoxicity assessment

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Abstract In this work, films based on bacterial cellulose nanofibers (BCNFs) incorporating gelatin hydrolysate (GH) from tilapia skin were produced. The effect of plasticizer (sorbitol or glycerol) and GH incorporation was evaluated on the physical–chemical and optical properties of films. BCNFs were produced using bacterial cellulose obtained from Hestrin and Schramm (HS) medium (BCNF-HS) or cashew apple juice (BCNF-CM), which was studied as an alternative to HS. Films with sorbitol showed the best properties and were selected for further characterization, using 40% (w/w) of BCNF-HS, 40% (w/w) of GH and 20% (w/w) of sorbitol (BCNF-HS-S-GH films). These films exhibited an antioxidant activity of 7.8 µmols Trolox Eq/g film, a water vapor permeability (WVP) of 1.6 g.mm/kPa.h.m^2 and an Young's modulus of 0.57 GPa. Films produced with BCNFs obtained from cashew apple juice revealed enhanced tensile strength, elongation at break, and thermal stability. Caco-2 cells' viability after incubation with BCNF-based films incorporating GH was evaluated and showed non-cytotoxicity, reinforcing the safety of the developed materials and their potential use in food applications.

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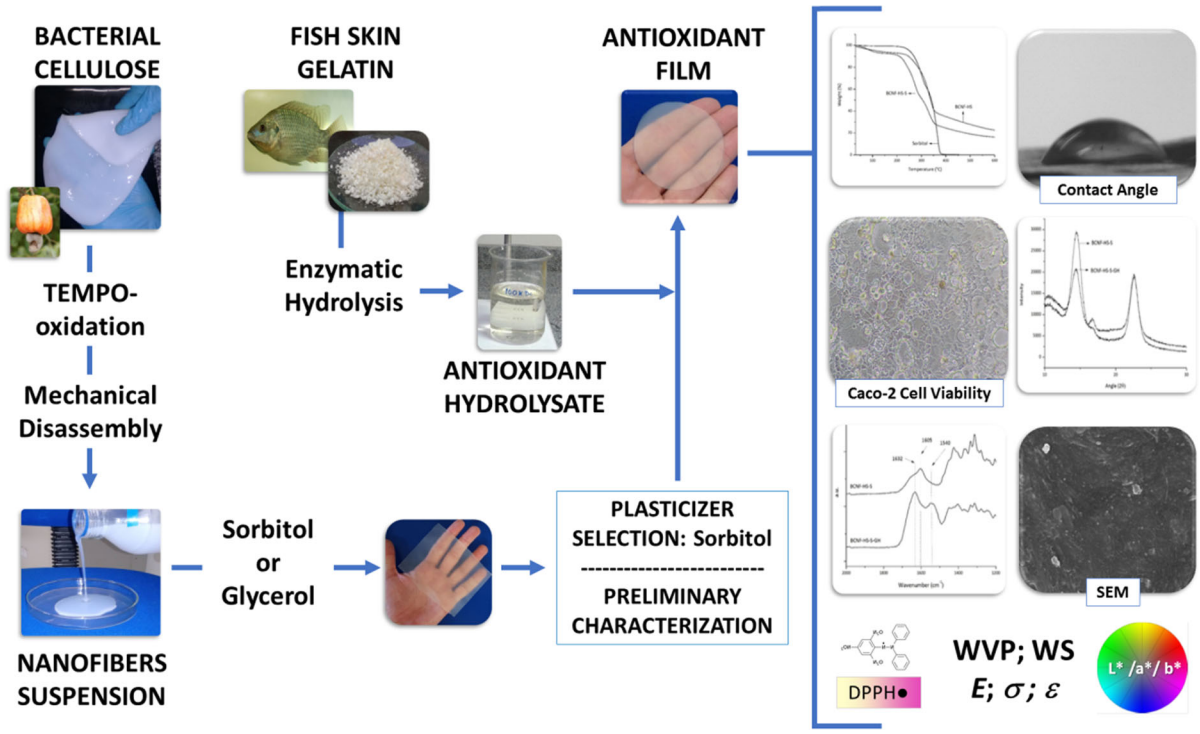
Graphical abstract



Keywords Biocomposite · Antioxidant · Plasticizer · Bioactive film · Cashew apple juice · Gelatin hydrolysate

Introduction

Bioactive edible films and coatings have been reported by many authors (Padrão et al. 2016; Salgado et al. 2015) to preserve and improve food quality and safety. Their use can increase the food shelf life through the decrease of microbiological growth and oxidation phenomena by controlling the release of bioactive agents on the food surface. In this context, antioxidants, antimicrobials, nutrients, nutraceuticals, flavours and dyes can be added to edible films and coatings, bringing different and additional functionalities (Coma et al. 2008; Perez et al. 2012). Natural polymers and bioactive compounds are the preferential choice for these applications, addressing the consumers demand for natural and safe food products complying with the regulator requests, and also meeting environmental issues related with disposal of non-biodegradable and non-renewable packaging (Atarés and Chiralt 2016; Bauer et al. 2001). It is nevertheless important to evaluate the toxicity of the produced films, since the production process may convert some components into non-safe substances (Montero et al. 2017).

Proteins from marine source have been explored to produce hydrolysates with antioxidant activity (Sila and Bougatef 2016). Tilapia (*Oreochromis niloticus*) is one of the several fish species studied for this purpose (Sampaio et al. 2017). These bioactive peptides, obtained by chemical or enzymatic hydrolysis of proteins, have been studied to develop bioactive films exploring their functional properties, such as antioxidant activity (Chi et al. 2015; Genskowsky et al. 2015; Martínez-Alvarez et al. 2015; Perez et al. 2012; Sun et al. 2015; Zhang 2016). Despite this, only few studies evaluated the potential of using antioxidant peptides in bacterial cellulose films for food applications (Han and Aristippos 2005; Lin et al. 2015a, b; Nguyen et al. 2008; Samaranayaka et al. 2011).

Bacterial cellulose (BC) is an extracellular polysaccharide excreted by bacteria from the genre

Komagataeibacter (previously named *Gluconacetobacter*) (Yamada et al. 2012), chemically identical to the vegetal cellulose but without the presence of lignin or hemicelluloses. BC is a promising polysaccharide for application in the food industry due to its high purity, food grade status, hydrophilicity, high sorption capacity of liquids and flexibility to be molded, colored and flavored. Moreover, BC can also be used as food additive to increase thermal stability, as texturizer and fat replaces (Shi et al. 2014; Ullah et al. 2016). Indeed, BC is a traditional dessert in Asia, particularly in the Philippines, known as 'Nata de Coco', mainly produced from coconut water and pineapple juice by simple static fermentation being independent of seasonal factors (Chawla et al. 2009).

The Hestrin & Schramm (HS) culture medium is usually used for BC production (Hestrin and Schramm 1954), although other synthetic media have been developed (Mohammadkazemi et al. 2015). Several studies have evaluated low-cost media for BC production, such as fruit juices, syrups, molasses, coconut water, glycerol, waste products and others (Jahan et al. 2017; Jozala et al. 2016; Chawla 2009; Duarte et al. 2015; Nascimento et al. 2016; Lima et al. 2017). The cashew crop peduncle (*Anacardium occidentale*, L.), a waste from cashew nuts processing, release citric acid and sugar when squeezed and despite the efforts, only 10% of the total waste produced is re-used in food manufacturing (Silveira et al. 2012). One of the possibilities for the valorization is to use this waste as an alternative culture medium for BC production (Duarte et al. 2015).

Currently, natural nanofibers are widely studied to produce high-performance biodegradable materials, such as reinforced packaging, composites, electronic paper, optical membranes, barrier films, flame-resistant materials, and other high-tech materials (Isogai et al. 2011; Kargarzh et al. 2017). One of the sources of natural nanofibers is cellulose, in which the chemical treatment and/or mechanical deconstruction can be applied, to obtain cellulose fibers in nanoscale. Mechanical treatments include homogenization, high-pressure microfluidization, mechanical deconstruction in a blender, and ultrasonication (Coelho et al. 2018; Lavoine et al. 2012). Chemical treatments, such as N-oxyl-2,2,6,6-tetramethylpiperidine (TEMPO)-mediated oxidation, carboxymethylation, acetylation, and/or enzymatic treatment usually precedes mechanical deconstruction. TEMPO-mediated oxidation has been used to obtain more uniform cellulose fibers after mechanical treatment, facilitating the separation of aggregated micro/nanostructures and enhancing stability. TEMPO-mediated oxidation provides advantages when compared to other chemical treatments, such as maintenance of the crystallinity and morphology of the fibers, fast reaction, mild process conditions (temperature and pressure), chemical selectivity and non-reactivity/sensibility to light, air or moisture (Isogai et al. 2011). Although many processes to obtain vegetal cellulose nanofibers had been widely studied, few report the production of nanofibers based on bacterial cellulose (BCNF) and their use in obtaining films (Saito et al. 2006; Tsalagkas et al. 2016).

In this context, this work aimed to produce and characterize BCNF based-films incorporating tilapia skin gelatin hydrolysate (GH). The effect of plasticizers (sorbitol or glycerol) and GH on properties of BCNF-based films was evaluated. Also, the influence of the BCNF source on the characteristics of BCNF-based films obtained was investigated by substituting BCNFs derived from fermentation in synthetic medium for BCNFs from an alternative medium (cashew apple juice). Selected films were characterized by water vapor permeability, water solubility, mechanical properties, thermal properties, antioxidant activity, including the cytotoxicity evaluation using a human intestinal epithelial cell line.

Materials and methods

Materials

Bacteriological agar, casein peptone, and yeast extract powder were purchased from Difco™ (Becton, Dickinson and Company, Sparks, MD, USA). D-Mannitol, citric acid hydrate, anhydrous D (+) glucose, sodium dihydrogen phosphate anhydrous, hydrogen peroxide, hydrochloric acid, sodium hydroxide, 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), sodium bromide, potassium bromide, sodium hypochlorite, magnesium nitrate, glycerol, and sorbitol were purchased from Sigma-Aldrich (Brazil). Resazurin, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and sodium pyruvate were purchased from Sigma-Aldrich (St Louis, MO, USA). Alcalase 2.4L was a

donation from Novozymes® (Bagsvaerd, Denmark). The cashew apple juice was collected in the Embrapa Tropical Agroindustry experimental field in Pacajus city (Ceará, Brazil). MEM (Minimum Essential Medium) was purchased from Thermo Scientific (Stafford, United Kingdom). Penicillin/streptomycin, fetal bovine serum (FBS) and non-essential amino-acids were purchased from Millipore (Darmstadt, Germany).

Preparation of bacterial cellulose nanofibers

Komagataeibacter xylinus ATCC 53582 strain, kept at -18 °C in HS medium with glycerol 20% (w/v), was activated in mannitol broth (5.0 g/L of yeast extract, 3.0 g/L of peptone and 2.5 g/L of D-mannitol) and statically cultured at 30 °C for 2 days. The culture was propagated by inoculation of 3% (v/v) from mannitol broth to HS medium (Hestrin and Schramm 1954) at 30 °C for 24 h and propagated for two more days to create the inoculum. BC membranes used in this study were obtained by bacteria cultivation in HS medium at pH 6.0 or cashew apple juice medium (CM) with 100 g/L of total reducing sugar at pH 6.0. Both culture media were autoclaved at 121 °C for 15 min, before inoculation. The BC was produced in glass bowl (25 × 27 cm) containing 500 mL of medium with 3% of inoculum (v/v), incubated at 30 °C under static conditions for 10 days. After fermentation, BC membranes were harvested and purified by alkali treatment prior to dry weight determination. For this purpose, impure films were washed in hot water (90 °C), following immersion into an alkaline solution (1 M NaOH + 1% H_2O_2 v/v) at 80 C for 1 h. Finally, BC membranes were rinsed with distilled water until pH 7.0. BC membranes from HS medium or CM are further referred as BC-HS and BC-CM, respectively.

To produce oxidized bacterial cellulose, BC-HS or BC-CM membranes were dried (at 50 °C), triturated and oxidized by TEMPO following a methodology developed by Saito et al. (2007), with few modifications. Dry films were suspended in distilled water (100 mL per gram of film) containing TEMPO and KBr (0.016 g and 0.1 g per gram of film, respectively). The oxidation was initiated by addition of NaClO 11% solution (NaClO 5.0 mmol/g on a dry BC basis) to the suspension, at 25 °C, under stirring (500 rpm). After 20 min, the pH was adjusted to 10.0 by adding 0.5 M NaOH solution, and the suspension was kept under magnetic stirring for further 2 h. The suspension was then washed up to pH 7.0. BCNFs from BC-HS or BC-CM media are further referred as BCNF-HS and BCNF-CM, respectively. After that, BCNFs (BCNF-HS or BCNF-CM) were mechanically treated in high speed blender (VITAMIX® 5200 Standard—Getting Started) at 25 000 rpm for 30 min, in three cycles (10 min each). The nanometric scale of the BCNF suspension was confirmed by transmission electron microscopy (TEM) analysis (data not shown).

Production of gelatin hydrolysate (GH) from fish skin

Tilapia skins received from filet processing unit (Fortaleza, Brazil) were immediately frozen until processing. The procedure defined by Sampaio et al. (2017) was used for gelatin extraction. Total protein in the dry gelatin powder was determined by Kjeldahl method (Kjeldahl 1883) using a factor of 5.55 to convert nitrogen into protein content.

To produce gelatin hydrolysate (GH), tilapia skin was dissolved in water (10 g/L) and hydrolyzed enzymatically using Alcalase 2.4L® (enzyme/substrate ratio of 5%) under controlled temperature (55 °C) and pH (6.0) for 3 h under magnetic stirring (300 rpm). The solution pH was adjusted with HCl or NaOH. The crude hydrolysate was heated at 80 °C for 10 min to stop the enzymatic reaction. After inactivation, the crude hydrolysate was concentrated to 90 g/L using a rotavapor (50 °C, 40 mbar), filtered through 0.22 µm membrane (Millex–hydrophilic PVDF, Brazil), and stored at − 18 °C, before use. Total protein content in GH was 88.5% (w/w) being 68.25% (w/w) hydrolyzed gelatin (< 100 kDa) and 20.25% (w/w) non-hydrolyzed gelatin (> 100 kDa).

To evaluate the peptide-sizes range obtained in the final hydrolysate, GH concentrated was filtered through a 10 kDa membrane and then through a 3 kDa membrane, using Amicon Ultra 4 mL centrifugal filters (Ultracel regenerated cellulose, Merck KGaA, Germany). The protein content, in each filtrate, was quantified by spectrophotometry, as described by Anthis and Clore (2013), using GH as standard. The antioxidant capacity of the gelatin hydrolysate was evaluated using DPPH method (Yang et al. 2009). The concentration of hydrolysate necessary to decrease the initial activity of DPPH· by 50% (IC_{50}) was 38.66 mg/mL. The % mass and antioxidant activity in each

Table 1 Composition (%, w/w) of BCNF-based films

Formulated films	Components (%, w/w)		
	BCNF	Plasticizer (sorbitol or glycerol)	Gelatin hydrolysate (GH)
BCNF-HS	100.0	0	0
BCNF-HS-S or BCNF-HS-G	66.7	33.3	0
BCNF-HS-S-GH or BCNF-CM-S-GH	40.0	20.0	40.0

BCNF-HS-S (composed of BCNFs from HS medium and sorbitol), BCNF-HS-G (composed of BCNFs from HS medium and glycerol), BCNF-HS-S-GH (composed of BCNFs from HS medium, sorbitol and gelatin hydrolysate) or BCNF-CM-S-GH (composed of BCNFs from cashew juice medium, sorbitol and gelatin hydrolysate)

fraction was: 68.63% and IC_{50} 25.07 mg/mL for < 10 kDa fraction and 46.52% and IC_{50} 32.97 mg/mL for < 3 kDa fraction, respectively.

Hydrolysate incorporation within BCNF-based films

To produce BCNF-based films incorporating gelatin hydrolysate, 400 mL of film-forming solution, composed by BCNF suspension (BCNF-HS or BCNF-CM) (1%, w/v) and GH solution (1%, w/v), at 1:1 (v/v) ratio, was dried in a polyethylene tray (20 × 15 cm) with polypropylene plastic sheet underneath the liquid, at 50 °C for 48 h. For plasticizer application, 1 g of sorbitol or glycerol was previously dissolved in 200 mL of 1% (w/v) GH. Control films were produced dissolving sorbitol or glycerol in water instead of in GH, and after being mixed with the BCNF suspension. The film-forming solutions were degassed before pouring the mixture onto the polyethylene tray. The GH amount used in the film-forming solution was previously selected in exploratory tests (data not shown) where the antioxidant activity was evaluated. The composition of the dry films is described in Table 1.

Characterization of BCNF-based films

Mechanical properties

Tensile tests (at least eight replicates) were conducted following ASTM D882-97 methodology (ASTM 1997) with few modifications. The films were cut (12.6 × 1.2 cm) and stored in a desiccator with controlled humidity (with magnesium nitrate 50–55%) for 48 h. The measurements were performed in a Universal Testing Machine (EMIC DL3000), with 12.5 mm/min draw speed, 50 kgf load cell employed and 10 cm of initial gap.

Thermogravimetric analysis (TGA)

Thermogravimetric analysis of films was performed on a thermogravimetric analyzer (Shimadzu, TGA STA 6000), using 10 mg of dried samples in the range of 0–600 °C under a nitrogen atmosphere (40 mL/min) at 10 °C/min of heating rate. Maximum degradation temperature (T_{max}), initial degradation temperature (T_{onset}) and final degradation temperature (T_{ofset}) values were identified for the evaluation of thermal stability.

Morphology

The morphology of films was evaluated by Scanning Electron Microscopy (SEM). For SEM analysis, the films were mounted on stubs, coated with gold and observed in a MEV TESCAN Scanning Electron Microscope, under 15 kV voltage acceleration.

Water vapor permeability (WVP) and water solubility (WS)

The water vapor permeability (WVP) of films was determined following E96-05 method with few modifications (ASTM 1989). Five circular samples (area of 22 cm^2) were sealed as patches onto acrylic permeation cells containing 6 mL of distilled water. The cells were placed in a desiccator with steady flow of dried air and weighed on an analytical balance at different time points up to 24 h. Eight measurements

were performed in each circular sample and the average was considered.

The water solubility (WS) was determined following the methodology reported by Soni et al. (2016), with few modifications. The films (2 × 2 cm) were dried (103 °C, 24 h) and weighted. Each film was immersed in 50 mL of distilled water and magnetically stirred (150 rpm) for 24 h at 25 °C. After that, the remaining film was filtered in paper (porosity 8 μm), dried (103 °C, 24 h) and weighted. Results were express in percentage of mass loss.

Contact angle

The contact angle was measured in a face contact angle meter (OCA 20, Dataphysics, Germany) at 19 °C, by the sessile drop method (Albuquerque et al. 2017) using a syringe equipped with a needle with internal diameter of 0.713 mm (Hamilton, Switzerland). Contact angle measurements were performed 120 s after placing a drop (3 μL) of ultrapure water on the film surface. Images were captured by CCD video camera (resolution of 752 × 582 pixels) and processed by C20 software. At least 12 measurements were performed for each sample to obtain an average value.

Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra were recorded with a Bruker FT-IR VERTEX 80/80v (Boston, USA) in Attenuated Total Reflectance mode (ATR) with a platinum crystal accessory in the wavelength range: 4000–400 cm^{-1}, using 16 scans at a resolution of 4 cm^{-1}. Before analysis, an open beam background spectrum was recorded, as a blank.

X-ray diffraction (XRD)

X-ray diffraction patterns of the films were analyzed between $2\theta = 10°$ and $2\theta = 50°$ with a step size $2\theta = 0.02°$ and recorded 14 pts/s with a Cu source, X-ray tube ($\lambda = 1.54056$ Å) at 45 kV and 40 mA in an X-ray diffraction instrument (X'Pert^3 Powder, PANalytical, Almelo, Netherlands). The crystallinity degree (C_D) was calculated according to Segal et al. (1959).

Transparency and color

Transparency was determined according to Podshivalov et al. (2017), using a solid module of Varian spectrophotometer (Cary 50). Color of films was measured with a Colorimeter (Hunter Lab System, by a Chroma Meter CR-300–Konica Minolta Sensing Inc., Osaka, Japan) and results were expressed in *L**, *a**, *b** parameters.

Film thickness

The films thickness was measured using a digital micrometer (Mitutoyo—QuantuMike IP65, Japan). Five measures were performed in each test film and the average was considered for Young's modulus (E), tensile strength (σ), and elongation at break (ε) calculations.

Antioxidant activity (AA)

Antioxidant activity (AA) of films was determined using the DPPH method (Yang et al. 2009) adapted for films. Each film (0.7 × 0.7 cm, corresponding to ~ 9 mg) was immersed in 1.5 mL of deionized water and stirred in an orbital shaker (100 rpm, 15 min). Afterwards, 1.5 mL of the DPPH solution (0.1 mM, in ethanol 95%) was added in the dark, under stirring at 100 rpm for 30 min before spectrophotometric reading (517 nm). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard and deionized water as a blank. The AA was expressed in trolox equivalent (μmols Trolox Eq by gram of protein).

Cytotoxicity assessment using intestinal epithelial cells

Cell culture

The human colon carcinoma Caco-2 cell line (ATCC, HTB-37) was used at passages 25–40. Caco-2 cells were grown in culture flasks containing Minimum Essential Medium (MEM), supplemented with 20% (v/v) fetal bovine serum, 0.11 mg/mL sodium pyruvate, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin/streptomycin. The cells were kept at 37 °C in 5% CO_2 water-saturated atmosphere.

Cytotoxicity—cell viability assessment using the resazurin assay

The resazurin assay was used to assess the cellular viability of Caco-2, after incubation with BCNF-based films (Ahmed et al. 1994; Nowak et al. 2017; Xu et al. 2015). Resazurin dye is a cell permeable redox indicator that has been broadly used as an indicator of cell viability in proliferation and cytotoxicity assays (Nociari et al. 1998). Viable cells with active metabolism can reduce resazurin into the resorufin product, which is pink and fluorescent. The quantity of resorufin produced is proportional to the number of viable cells.

Caco-2 cells were seeded onto 96-wells plate at a density of 10,000 cells per well and left adhering overnight. After adhesion, the culture medium was removed and replaced by the BCNF-based films or individual components, diluted in the culture medium.

The BCNF-based films were first dispersed in ultrapure water (milli-Q) using the Ultra-Turrax homogenizer at 3000 rpm for 1 min, then sonicated in ultrasonic bath for 15 min (37 kHz and 104 Watt) and finally exposed to ultraviolet lamp during 30 min for sterilization. The water dispersions of BCNF-based films or the control compounds were diluted in the culture medium (10%, v/v) to obtain the test concentrations. A negative control was performed using cells growing in the culture medium (considered as 100% cell viability). DMSO (40% v/v) was used as a positive control. The samples were incubated for 24 h or 48 h with 10% (v/v) resazurin solution (final concentration 0.01 mg/mL). Resazurin was added simultaneously with samples, since it is not toxic providing adequate sensitivity (Ahmed et al. 1994; Xu et al. 2015). For all samples, a blank was performed using samples diluted in the culture medium and incubated with resazurin (without cells).

The fluorescence intensity, that is proportional to the cell viability, was measured using a Microplate Fluorescence Reader (Synergy, BioTek H1, USA) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The % cell viability was expressed as fluorescence of cells treated with samples compared to the fluorescence of cells growing in the culture medium (100% cell viability) as follows:

$$\%Cell\ Viability = \left[\frac{F_{TC} - F_S}{F_C - F_{CM}}\right] \times 100$$

where F_{TC} is the fluorescence of treated cells, F_S is the fluorescence of sample in the culture medium (without cells), F_C is the fluorescence of cells growing in the culture medium, F_{CM} is the fluorescence of culture medium (without cells).

Statistical analysis

The statistical analysis was carried out using the software Origin Pro 9.0 (OriginLab Corporation). The statistical significance of the evaluated data was analyzed by one-way analysis of variance (ANOVA) and the Tukey's test with significance level (α) = 0.1.

Results and discussion

Effect of plasticizers on properties of BCNF-based films

The effect of plasticizers was evaluated on the mechanical properties, water vapor permeability, water solubility, crystallinity and thermal behavior of BCNF-HS based films (Table 2). The plasticizers sorbitol and glycerol were added to improve mechanical properties and facilitate handling of the films (BCNF-HS-S and BCNF-HS-G, respectively). Once the BCNF-HS films exhibited high brittleness behavior, the mechanical tests were performed only with those containing plasticizers. Films with glycerol exhibited a Young's modulus (E) 28.16% lower and an elongation at break (ε) 37.20% higher than the films using sorbitol as plasticizer (Table 2). This result may be related to the superior capacity of glycerol to act as a plasticizer, which is explained by its lower molecular weight and ability to improve water incorporation (Csiszár and Nagy 2017; Cerqueira et al. 2012a). The E reduction and ε increase observed for glycerol containing films when compared to sorbitol, were also observed by Thomazine et al. (2006) for gelatin-based films.

TEMPO-oxidized cellulose nanofibers provide good mechanical properties assigned to the dense and strong cellulose network (Rodinova et al. 2012; Wu and Cheng 2017). BCNF-HS-S films exhibited higher tensile strength than several polymeric films reported in the literature, such as alginate/cellulose nanofibers (Deepa et al. 2016), starch (Moreno et al.

2015; Piñeros-Hernandez et al. 2017; Yunos and Rahman 2011), kappa-carrageenan (Farhan and Hani 2017), gelatin (Thomazine et al. 2006; Tongnuanchan et al. 2012, 2015), gelatin/dialdehyde carboxymethyl cellulose (Mu et al. 2012), and chitosan (Cerqueira et al. 2012b; Soni et al. 2016). Regarding the elongation at break (ε), the value obtained for BCNF-HS-S films is higher than kappa-carrageenan films (Farhan and Hani 2017) and microfibrillar cellulose (Syverud and Stenius 2009).

Crystallinity degree (C_D) of unplasticized and plasticized films was similar (Table 2). X-ray diffraction profiles revealed high crystallinity degree, similar to those reported elsewhere for bacterial cellulose (Campano et al. 2016).

In the thermal behaviour it was found that the addition of sorbitol or glycerol into the BCNF-HS films reduced the T_{onset} from 252 to 221 °C or 172 °C, respectively, and leads to the appearance of new derivative-thermogravimetric (DTG) peaks (Fig. 1, Table 2). Multiple DTG peaks are assigned to the presence of non-cellulose compounds, such as proteins or metabolic compounds from microbial growth (Gea et al. 2011). Typical events of BC degradation were observed around 330 °C (Fig. 1) in all films, with or without plasticizers. Fukuzumi et al. (2009) and Fukuzumi et al. (2010) reported that films of TEMPO-oxidized cellulose nanofibers exhibited T_{onset} from 200 to 222 °C, therefore close to BCNF-HS-S value obtained in this study and lower than BCNF-HS.

The thermal stability is associated with plasticizers type and their interaction with the polymer. Films containing glycerol (BCNF-HS-G films) exhibit lower T_{onset} than those with sorbitol (BCNF-HS-S films) (Table 2). Sorbitol has more hydroxyl groups available to interact with cellulose by hydrogen bonds, leading to better thermal stability when compared to films with glycerol. It has been reported that glycerol exhibits a lower value of T_{onset} and DTG peak than oxidized cellulose, as observed in this work for the BCNF-HS-G films (Ciriminna et al. 2014; Gómez-Siurana et al. 2012; Yunos and Rahman 2011). With respect to the film containing sorbitol (BCNF-HS-S) it presents a lower T_{onset} (215 °C) than separated components (251 °C for BCNF-HS and 257 °C for sorbitol).

The water solubility (WS) gives an indication of the films' stability when they are in a high hydrophilic medium. The plasticizers have a great influence in the WS since BCNF-HS films present WS values close to

Table 2 Mechanical properties of BCNF-based films

Film	BCNF-HS	BCNF-HS-S	BCNF-HS-G	BCNF-HS-S-GH	BCNF-CM-S-GH
E (GPa)	**	$1.42^{a} \pm 0.13$	$1.02^{b} \pm 0.11$	$0.57^{c} \pm 0.05$	$0.50^{c} \pm 0.07$
σ (Mpa)	**	$87.04^{a} \pm 12.70$	$78.43^{a} \pm 10.02$	$26.27^{b} \pm 3.75$	$45.72^{c} \pm 7.71$
ε (%)	**	$12.47^{a} \pm 1.87$	$17.11^{b} \pm 2.29$	$9.60^{a} \pm 1.09$	$30.40^{c} \pm 7.49$
T_{onset} (°C)	252	221	172	206	256
DTG peaks (°C)	333	330/274/231	333/239/201	314/262/144	347/287
C_D (%)	84	82	83	75	82
WS (%)	n.d	$34.68^{a} \pm 6.10$	$69.58^{b} \pm 5.95$	$59.54^{c} \pm 5.00$	$78.46^{d} \pm 6.76$
WVP (g.mm/kPa.h.m^2)	$0.48^{a} \pm 0.03$	$0.93^{b} \pm 0.08$	$1.00^{b} \pm 0.12$	$1.54^{c} \pm 0.08$	$1.58^{c} \pm 0.14$
Contact angle (°)	$41.53^{a} \pm 5.62$	$65.72^{bc} \pm 10.70$	$71.72^{c} \pm 9.27$	$58.75^{ab} \pm 11.95$	$40.75^{a} \pm 7.32$
AA (μmols TEAC/g of protein)	n.d	n.d	n.d	$7.80^{a} \pm 1.60$	$7.29^{a} \pm 0.46$
T (n/a)	$0.18^{a} \pm 0.00$	$0.62^{b} \pm 0.01$	$1.00^{c} \pm 0.04$	$1.02^{c} \pm 0.02$	$1.00^{c} \pm 0.03$
*L**	$90.39^{ab} \pm 0.01$	$90.12^{a} \pm 0.36$	$93.2^{c} \pm 0.27$	$88.99^{b} \pm 0.82$	$87.74^{b} \pm 1.03$
*a**	$4.73^{ab} \pm 0.08$	$4.5^{c} \pm 0.08$	$-0.04^{d} \pm 0.09$	$5.05^{b} \pm 0.23$	$4.76^{a} \pm 0.11$
*b**	$6.73^{a} \pm 0.43$	$7.28^{a} \pm 1.08$	$10.68^{b} \pm 0.46$	$10.69^{b} \pm 2.57$	$12.33^{c} \pm 1.86$

Means in the same line with different superscript letters are significantly different ($P < 0.1$)

E Young's modulus, σ tensile strength, ε elongation at break, T_{onset} *and DTG peaks* thermal properties, C_D crystallinity degree, *WVP* water vapor permeability, *WS* water solubility, *AA* antioxidant activity, *T* transparency, color parameters (L*/a*/b*)

*The brittleness of films could not be analyzed

**Not performed and *n.d* Non-detected

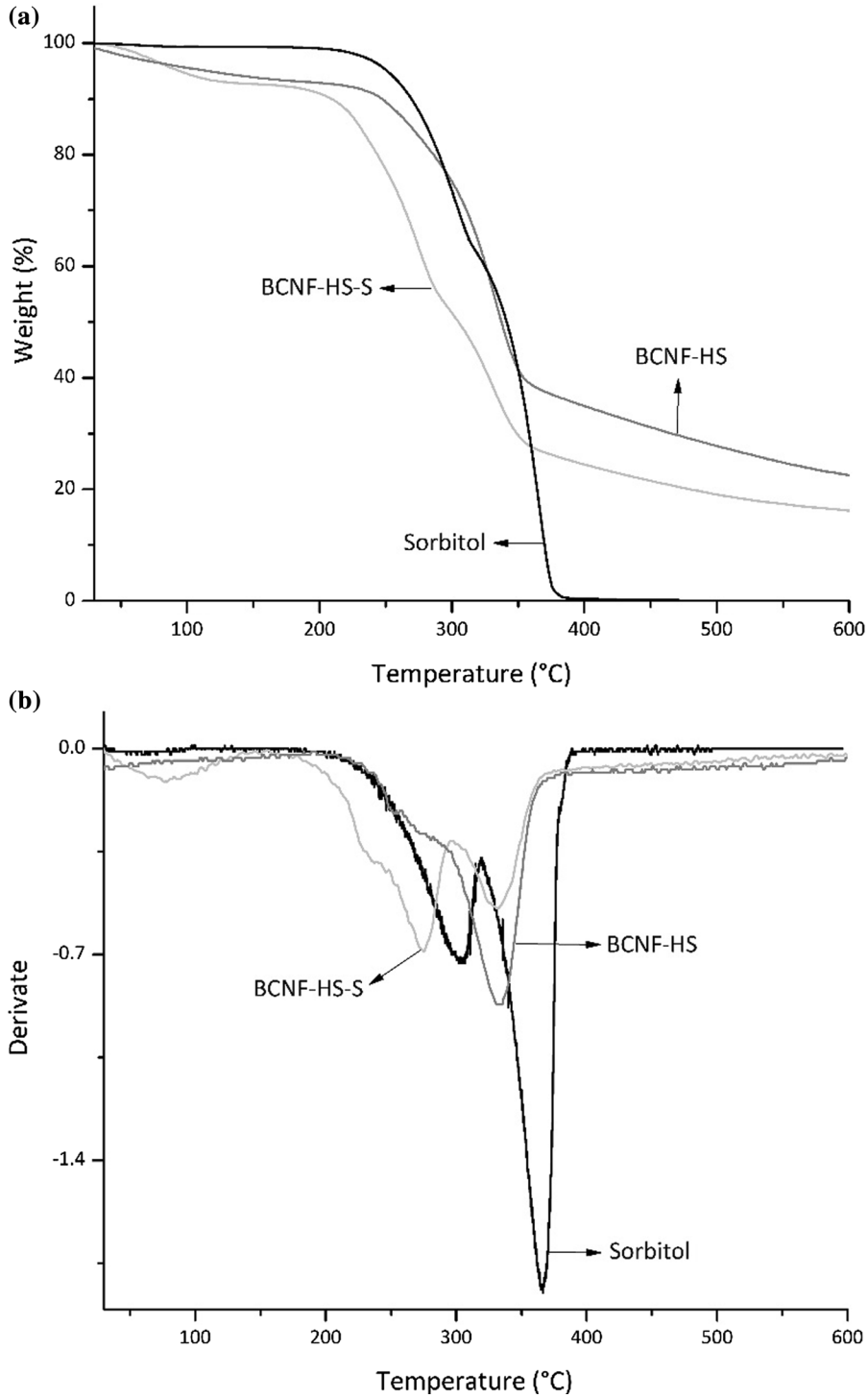


Fig. 1 **a** Thermogravimetric (TG) and **b** derivative thermogravimetric (DTG) curves of BCNF-based film containing sorbitol (BCNF-HS-S) and its components (sorbitol and BCNF-HS)

zero while plasticized films (BCNF-HS-S) exhibit a weight loss of 34.68%, close to the amount of sorbitol present in the films (Table 1), suggesting that it is totally released during the WS assay. Meanwhile, BCNF-HS-G had a higher solubility (69.58%) when compared to BCNF-HS-S. Deepa et al. (2016) mentioned that the intensity of hydrogen bonding between the components of a composite influences its solubility and water resistance which in this case can be justified by the plasticizer type, as previously explained.

Regarding the WVP values, both films, BCNF-HS-S and BCNF-HS-G, exhibited similar values of WVP ($P > 0.1$), which are 93.75% and 108.33% higher, than the films without plasticizer (BCNF-HS) (Table 2). This effect is explained by the hydrophilic behavior of plasticizers, which due their hydroxyl groups and low molecular weight exhibit capacity to create regions of higher water mobility in polymer and thus contributing to the increase of water diffusion, hence increasing WVP (Cao et al. 2009). The WVP increase was also observed by Farhan and Hani (2017) after addition of sorbitol or glycerol into carrageenan films and by Nur Hanani et al. (2013) in gelatin films.

The hydrophilicity of films was confirmed by measuring the contact angle. All films exhibited contact angles lower than 90° revealing their hydrophilic feature. After plasticization, regardless of the plasticizer used, an increase in the contact angle was observed. This may be related to the formation of hydrogen bonding between plasticizers and cellulose, leading to a reduction in the number of exposed hydroxyl groups that previously remained free for interactions with water as previously reported by Cao et al. (2009).

BCNF-based films containing sorbitol showed the higher E, lower WS and higher thermal stability. Based on these results, the BCNF-based films containing sorbitol were selected for the further incorporation of gelatin hydrolysate (GH).

Characterization of BCNF-based films incorporating gelatin hydrolysate

Thermal and mechanical properties

The effect of gelatin hydrolysate incorporation into the BCNF-HS-S films on the thermal properties of films was evaluated. Figure 2 shows the thermogravimetric (TG) and derivative thermogravimetric (DTG) curves of films containing GH in comparison with films without GH. The T_{onset} was reduced from 221 to 206 °C, after GH incorporation. Moreover, three different DTG peaks are detected (315, 262 and 144 °C) assigned to non-cellulose compounds (Table 2). In the literature, Hoque et al. (2011) observed a reduction on mechanical and thermal properties of gelatin/glycerol films when replacing gelatin with gelatin hydrolysate. This behaviour was explained by the protein structure breakdown caused by reducing protein–protein and matrix-protein interactions.

Regarding the mechanical properties, the incorporation of GH into the BCNF-HS-S films lead to the decrease of E by 59.85% and the tensile strength by 69.81%, reaching 0.57 GPa and 26.27 MPa, respectively (Table 2). The addition of peptides or proteins have been reported to decrease σ due to the weakness of cohesive properties (Pei et al. 2013; Ferreira et al. 2009), in good agreement with the results obtained in this work. It has also been referred that peptides and hydrolysates can exhibit a plasticizer effect (Nuanmano et al. 2015; Ferreira et al. 2009), which was not observed in this study since ε was not significantly modified. Each particular combination of matrix, plasticizer and protein hydrolysate yields different properties according to the strength of the intermolecular interactions.

Although the incorporation of GH into BCNF-based films affected E and σ to some extent, the mechanical properties of the films produced remain suitable for several food applications. In fact, BCNF-HS-S-GH films exhibited higher tensile strength than several polymeric films reported in the literature, such as starch (Moreno et al. 2015; Piñeros-Hernandez et al. 2017; Yunos and Rahman 2011), chitosan (Cerqueira et al. 2012b; Soni et al. 2016), gelatin/glycerol (Nur Hanani et al. 2013), fish skin gelatin/palm oil (Tongnuanchan et al. 2015), gelatin/dialdehyde carboxymethyl cellulose (Mu et al. 2012), and gelatin/glycerol/sorbitol (Thomazine et al. 2006). The E obtained is higher than gelatin/glycerol/sorbitol films reported by Thomazine et al. (2006).

Morphology

All BCNF-based films bear a similar dense appearance that prevents the visualization of nanofibers by SEM (Fig. 3). The drying expressively affected the nanofibers compaction that is essential to form strong films.

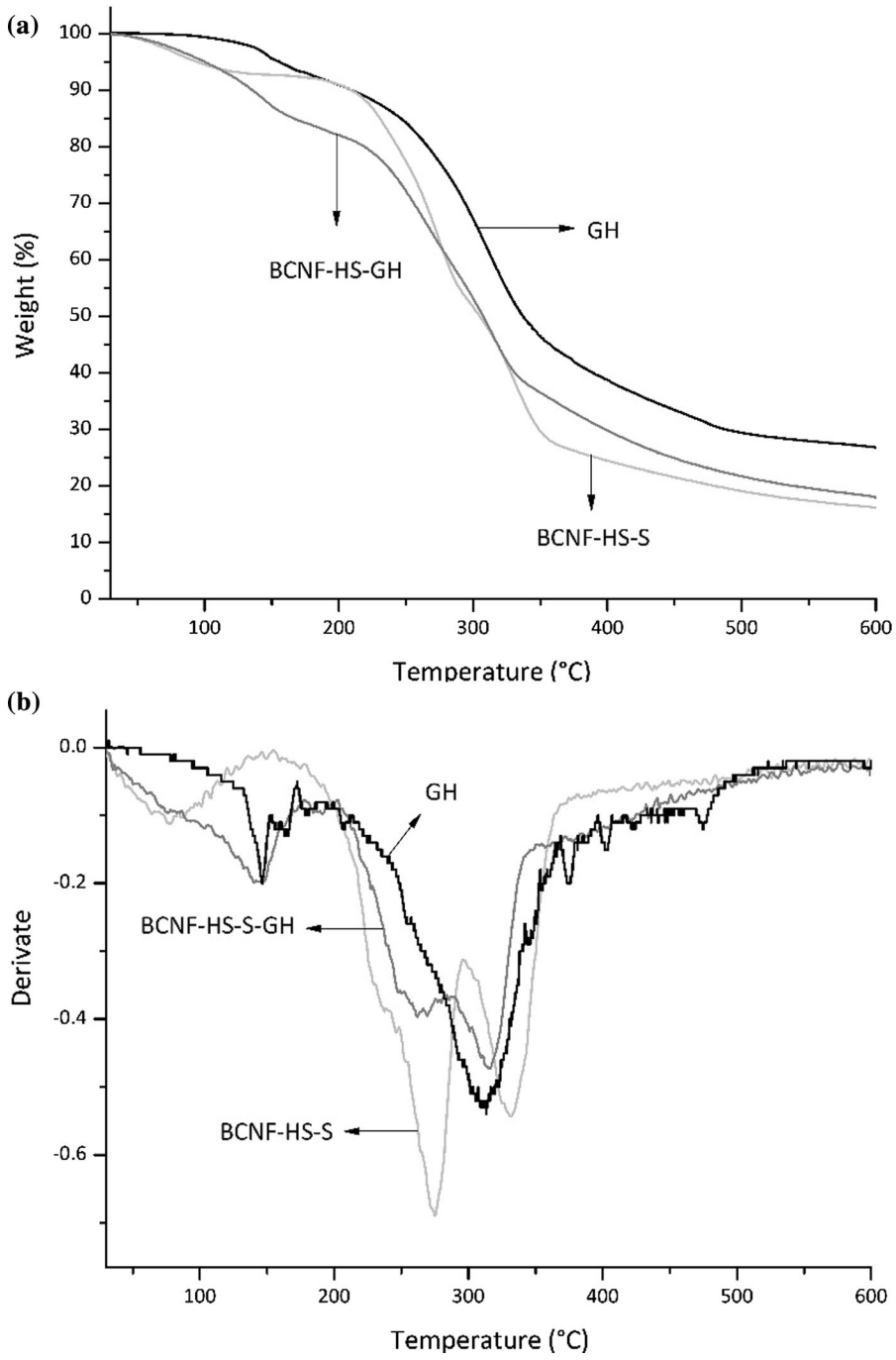


Fig. 2 **a** Thermogravimetric (TG) and **b** derivative thermogravimetric (DTG) curves of gelatin hydrolysate (GH) and BCNF-based films: BCNF-HS-S and BCNF-HS-S-GH

BCNF-HS-S-GH film appears to be less homogeneous than others due to the formation of aggregates, probably caused by GH proteins as observed by Lin et al. (2015a, b) and Gao et al. (2014) in bacterial cellulose/protein composites.

Water vapor permeability (WVP), water solubility (WS) and contact angle

The addition of gelatin hydrolysate (GH) to the films lead to an increase of water solubility when compared with films without GH, from 34.58% to 59.54% (Table 2). This value is close to the amount of sorbitol and GH present in the film composition (60%), indicating that these compounds are probably fully solubilized during the test. In fact, sorbitol and gelatin hydrolysates are soluble in water. Regarding the WVP of BCNF-HS-S-GH films, a value of 1.54 g.mm/kPa.h.m^2 was obtained, being the value 65.59% higher to the films without GH (0.93 g.mm/kPa.h.m^2, Table 2). The hydrophilicity and hygroscopicity of GH probably contributed to the higher WVP due to the increase of water–vapor adsorption promoted by polar groups (–COOH, –NH_2 and –OH) (Nuanmano et al. 2015). Hermansyah et al. (2013) reported the poor water barrier properties of gelatin and Ferreira et al. (2009) observed an increase in WVP of chitosan films after protein addition. The WVP observed for BCNF-HS-S-GH films is similar to the values presented for the WVP of other natural composites/polymers, such as alginate (Benavides et al. 2012; Olivas et al. 2008; Rhim, 2004), chitosan (Azeredo et al. 2010), gelatin (Santos et al. 2014), starch (Piñeros-Hernandez et al. 2017; Bertuzzi et al. 2007), whey protein and zein (Baldwin et al. 2012).

After GH incorporation, it was observed a reduction in the exhibited contact angle, which was 58.75° for BCNF-HS-S-GH versus 65.72° for BCNF-HS-S. The hydrophilicity was increased after GH incorporation probably due to excess of polar groups that weakened interactions between hydrophilic groups of cellulose, sorbitol and GH as reported by Cao et al. (2009) in their studies with gelatin films.

FTIR and X-ray diffraction (XRD)

FTIR spectra of studied films exhibited typical vibration bands of cellulose (Fig. 4 a, b): O–H bond stretching (3345 cm^{-1}), asymmetric stretching of

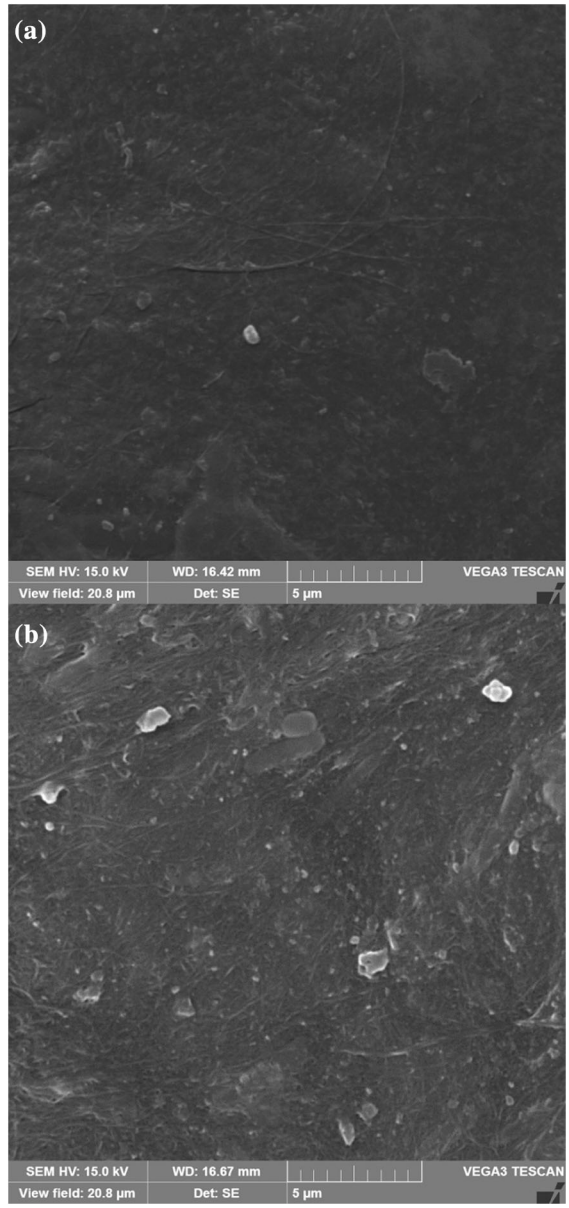


Fig. 3 Scanning electron microscopy images of BCNF-based films. **a** BCNF-HS-S and **b** BCNF-HS-S-GH

CH_2 (2920–2850 cm^{-1}), asymmetric angular deformation of C–H bonds (1426 cm^{-1}), symmetric angular deformation of C–H bonds (1360–1378 cm^{-1}), asymmetrical stretching of C–O–C glycosidic bonds (1160 cm^{-1}), stretching of C–OH and C–C–OH bonds for secondary and primary alcohols respectively (1107–1055 cm^{-1}), C-H bond bending or CH_2 stretching (900 cm^{-1}), and O–H out-of-plane bending (665 cm^{-1}). BCNF-HS-S-GH films presented an

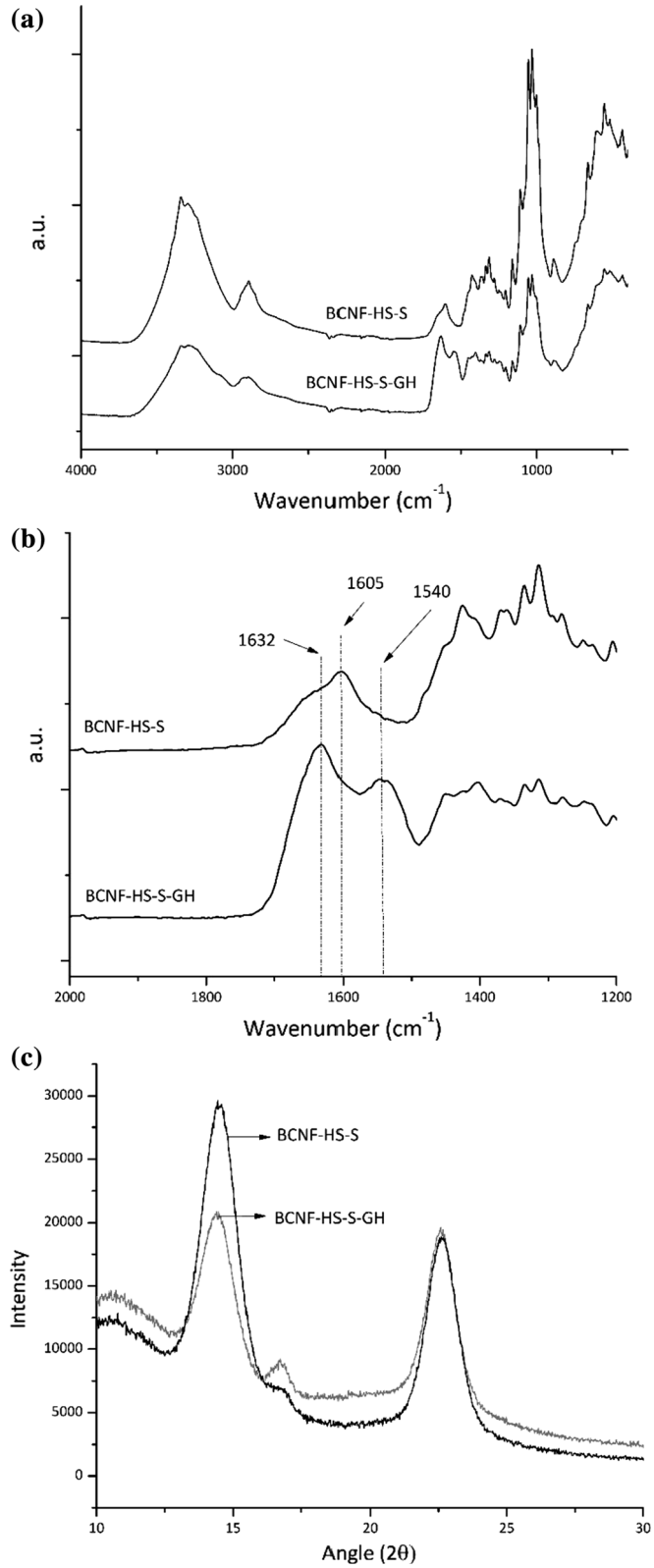


Fig. 4 **a**, **b** FTIR spectra and **c** X-ray diffractograms of BCNF-based films: BCNF-HS-S and BCNF-HS-S-GH

intense band at approximately 1700–1500 cm^{-1} related to nitrogen and protein structures: C=O stretching in type I amides (1632 cm^{-1}) and angular deformation in type II amides (1540 cm^{-1}) (Gea et al. 2011), due to the presence of GH. 1605 cm^{-1} band related to C=O stretching of COONa (Wu and Cheng 2017) in BCNF-HS and BCNF-HS-S films is slightly more intense than in BCNF-HS-S-GH which can be explained by the lower amount of TEMPO-oxidized cellulose (BCNFs) (Table 1).

The C_D value was slightly reduced (10.7%) in BCNF-HS-S-HG film when compared to BCNF-HS-S, although still high and characteristic of BC (Campano et al. 2016) (Fig. 4c). This expressive reduction may have occurred due to a possible interaction of the gelatin hydrolyzate added with the bacterial cellulose nanofibrils. Zhijiang and Guang (2010) also reported a reduction in the crystallinity index of BC films with addition of collagen. They assume that the interaction of collagen with BC must have disturbed the regular arrangement of BC molecule chains, which in turn can decrease the crystallinity index. Also, this rearrangement in the disposition of nanofibrils may have contributed to the reduction of mechanical properties of BCNF-HS-S-HG film versus BCNF-HS-S film (Table 2), discussed previously in item 3.2.1.

Transparency and color

BCNF-based films containing sorbitol and antioxidant hydrolysate presented higher transparency than films made of BCNFs only, probably due to a better packing of the film with additives filling the space between the fibers, hence impacting on the refraction of light. BCNF-HS-S-GH films exhibited a slightly yellower color (b^*) when compared to others (Table 2). This increase on yellow coloration in BCNF-based films with crude hydrolysates might be related to creamy yellowish color of hydrolyzed gelatin that may be related to the presence of compounds produced in the Maillard reaction, between carbonyl groups resultant of lipid oxidation (e.g. aldehydes and ketones) and amino groups of free amino acids or peptides (Schmid et al. 2013).

Effect of the BCNF source on the film properties

Aiming to investigate the potential of application of BC obtained in a medium of low cost culture, the cashew apple juice was selected as an alternative medium. Films containing sorbitol and gelatin hydrolyzate produced using BCNFs derived from the cashew apple juice medium (BCNF-CM-S-GH) exhibited WVP, E, transparency, contact angle, b^* color parameter, and antioxidant activity statistically similar to films of BCNFs derived from synthetic medium HS (BCNF-HS-S-GH), as shown in Table 2. Moreover, the tensile strength, elongation at break, crystallinity degree, T_{onset} and WS of BCNF-CM-S-GH were increased by 74.03, 216.66, 8.44, 24.27, and 31.76%, respectively, when compared to BCNF-HS-S-GH film. Recent findings in the literature have reported that changes in characteristics such as degree of polymerization and intermolecular arrangement of BC molecule chains, influenced by type of culture medium, contribute to differences in BC properties such as the mechanical properties and crystallinity (Campano et al. 2016; Zhijiang and Guang 2010). Thus, structural changes in BC produced in cashew medium must have contributed to the properties of BCNF-CM-S-GH films.

Cytotoxicity—cell viability through resazurin assay

The biocompatibility of materials is routinely evaluated using in vitro methodologies. Cell lines are often cultivated in contact with test materials, and after a variable period, the cellular metabolic activity, proliferation and/or death rates are measured (Gosslau 2016). In this study, a resazurin solution was used to assess the cellular viability of Caco-2 after incubation with BCNF-based films or with the respective controls.

As shown in Fig. 5, the BCNF-based films or the individual components (plasticizer, hydrolysate or bacterial cellulose nanofibers) had no effect on the metabolic activity of Caco-2 cells, irrespective of the incubation period. All samples exhibit high cell viability up to 48 h of incubation demonstrating the biocompatibility of the produced BCNF-based films. The use of BCNFs, as a carrier of bioactive compounds in food applications, turns the toxicological assessment mandatory. The results of this study indicate that the tested BCNF-based films by themselves are not cytotoxic, thus appropriate for use in food or packaging applications.

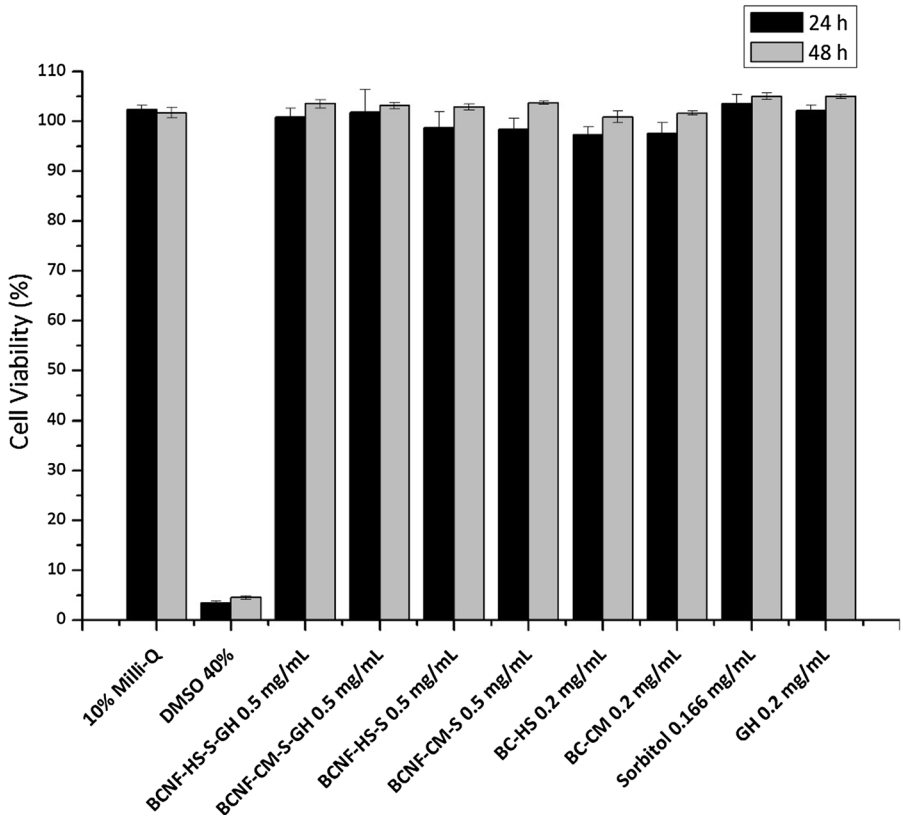


Fig. 5 Cellular viability of Caco-2 after incubation (24 and 48 h) with BCNF-based films or with the respective controls. Controls: (-) cells incubated with 10% milli-Q (negative control); (+) cells incubated with 40% of DMSO (positive control). Data shown mean ± SD, n = 8

Conclusions

Antioxidant films based on bacterial cellulose nanofibers (BCNFs), plasticizers (glycerol and sorbitol) and tilapia skin gelatin hydrolysate (GH) were successfully produced. Films plasticized with sorbitol exhibited better performance than glycerol, regarding water solubility, Young's modulus and thermal properties. Although, the incorporation of GH into BCNF-based films affected their properties, the present work demonstrated that the produced films present antioxidant activity and have improved characteristics compared to similar polymeric films reported in the literature. The replacement of BCNFs from synthetic culture medium by BCNFs from cashew apple juice medium, used for the films production, improved their tensile strength, elongation at break, thermal stability, and crystallinity degree. This implies that the BC of cashew medium allows the production of films with improved features arising as a low cost alternative to synthetic medium. The cytotoxicity assessment demonstrated that BCNF-based films are non-cytotoxic, reinforcing their potential for food applications.

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