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Production of bacterial cellulose by *Komagataeibacter xylinus* using mango waste as alternative culture medium

Producción de celulosa bacteriana por *Komagataeibacter xylinus* utilizando desperdicio de mango como fuente de carbono alternativa

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Abstract

Bacterial cellulose (BC) is a high value-added nano-structured biopolymer with important biomedical applications. Its biosynthesis from waste carbon sources might modify BC structure and properties, thus Mango pulp waste (MPW) was evaluated as an alternative culture medium for its production by the bacteria *Komagataeibacter xylinus*. The use of MPW could also decrease BC cost since the use pure sugars for its obtaining is expensive. The effect of different nitrogen sources and buffer addition to MPW-medium on the polymer yield was also investigated. Using MPW and yeast extract as a nitrogen source, a production of 6.32 g/L of BC was obtained after 16 days of static culture. BC was characterized by SEM, XRD, TGA, FTIR and Water holding capacity (WHC). Chemical structure and thermal degradation of BC produced from MPW were similar to those of BC obtained with pure sugars (350°C). Crystallinity index of BC produced in mango-based medium was lower (62.7 % vs. 77.2 %); WHC was higher (108.7 % vs. 88.7 %); and fiber diameter was smaller (98.8 nm vs. 50.6 nm).

Keywords: bacterial cellulose, *Komagataeibacter xylinus*, nanocellulose, mango waste.

Resumen

La celulosa bacteriana (CB) es un biopolímero nanoestructurado de alto valor agregado en aplicaciones biomédicas. Su biosíntesis a partir de fuentes de carbono alternativas podría modificar la estructura tridimensional de la membrana obtenida en cultivo estático y con ello, algunas de las propiedades de la CB, por lo que se evaluó la pulpa de mango de desperdicio (PMD) como medio de cultivo para su producción mediante el microorganismo *Komagataeibacter xylinus*. Se investigó el efecto de diferentes fuentes de nitrógeno y la adición de una solución buffer de citrato y fosfato al medio de PMD sobre el rendimiento final. Usando la PMD y extracto de levadura como fuente de nitrógeno, se obtuvo una producción de 6.32 g en peso seco de CB/L después de 16 días de cultivo estático. Se caracterizó la CB por SEM, XRD, TGA, FTIR y capacidad de retención de agua (WHC). La estructura química y la degradación térmica de la CB producida a partir de PMD fueron similares a la CB obtenida a partir de azúcares puros (glucosa, fructosa y sacarosa). El índice de cristalinidad de la CB producida en medio PMD (62.7 % vs. 77.2 %); la capacidad de retención de agua (WHC) fue mayor para la CB a partir de PMD (108.7 % vs. 88.7 %); y el diámetro de fibra fue menor (98.8 nm vs. 50.6 nm).

Palabras clave: celulosa bacteriana, *Komagataeibacter xylinus*, nanocelulosa, desperdicio de mango.

1 Introduction

Cellulose from bacteria is a nano-structured biopolymer that has taken importance and aroused the interest in its production and use for the development of products with high added value (Iguchi *et al*., 2000). BC is synthesized by different microorganisms, being *Komagataeibacter xylinus* one of most efficient producers (Yamada *et al*., 2012). This microorganism in static conditions of culture forms films as a flotation mechanism to stay in the liquid/air interface, from where it obtains the oxygen necessary for its own growth. The film is also a physical barrier that protects the bacteria from the UV radiation and the toxic compounds, at the same time it increases its ability to colonize substrates and allows it to retain humidity (Santos *et al*., 2016).

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The biocompatibility of BC has been widely studied and reported (Helenius *et al*., 2006), however, there are few commercial applications that currently exist. Among the products that are commercialized for medical and dental applications we can find the Biofill® and the Gengiflex®; one is used for wound dressing and the other one as a material used in dental implants, respectively. Other uses of BC, are *i.e*., as a temporary skin substitute with antimicrobial activity (mixing with silver nanoparticles) and for the treatment of wounds (Maneerung *et al*., 2008), burns, ulcers and abrasions in the epidermis (Cai *et al*., 2009); vascular implants for microsurgery (Esguerra *et al*., 2010); tissue engineering support materials as it because of it facilitates a cellular proliferation (Petersen and Gatenholm, 2011). Due to BC characteristics, another potential applications are as reinforcement in composites, in combination with hydroxyapatite for bone tissue replacement, with polyvinyl alcohol (PVA) for corneal implants, for immobilization of enzymes and other biomolecules and for controlled drugs release, among others (Esguerra *et al*., 2010; Bolio-López *et al*., 2011; Gutierrez *et al*., 2018).

Despite the properties and the potential uses of BC, large-scale production still presents difficulties due to the low productivity of current fermentation strategies and high production costs (Tsouko *et al*., 2015; Molina-Ramírez *et al*., 2018). It has been reported that the culture medium for BC production is responsible for up to 65% of the total cost of the process (Jozala *et al*., 2014). The culture media for the production of BC contain carbon, nitrogen and other macro and micronutrients necessary for the growth of the microorganism, being the most common Hestrim and Schramm (HS). There are numerous modifications to this medium, depending on the purpose of the study (Mohammadkazemi *et al*., 2015; Costa *et al*., 2017). In all cases, changes in the components of the medium affect microbial growth and BC production, either directly or indirectly by the formation of inhibitory metabolites (Lee *et al*., 2014; Sainz *et al*., 2017). Sometimes, a complex medium supplies amino acids and vitamins that improve cell growth and biopolymer production (Lee *et al*., 2014).

In recent years, there is a great interest in the use of agro-industrial or agro-food wastes to obtain diverse high value-added products (Pérez-Cadena *et al*., 2018; Simas-Díaz *et al*., 2018; Espinel-Rios *et al*., 2019). New technologies have been developed that use agroalimentary and industrial waste in the formulation of alternative culture media for the production of BC (Kongruang, 2008), such as pineapple and sugarcane juice (Castro *et al*., 2011); grape bagasse residue from wine production (Vazquez *et al*., 2013); apple waste from cider production (Urbina *et al*., 2017); among others, which have potential to be used as carbon sources for the formulation of culture media for biotechnological processes.

Mango (*Mangifera indica* L.) is a highly demanded fruit by consumers due to its good sensorial and nutritional properties (Mercado-Mercado *et al*., 2019). It is produced in many countries around the world being India, China, Thailand, Indonesia and Mexico the top five producing countries with yields from 2,000,000 to 18,000,000 ton per year (FAO, 2017). However, according to The UN Food and Agriculture Organization every year around to 55 % (1,100,000 ton) of mango production goes to waste in many countries, mainly associated with failures related to logistics and infrastructure to take their products from field to the national and international market. This agroindustrial waste is rich in sugars, therefore can be a raw material with great potential to be used as a carbon source in biotechnological processes, and in this case for the production of a high value-added polymer such as bacterial cellulose (BC).

The aim of this research was to evaluate the production of BC in static culture of *Komagataeibacter xylinus* using a medium based on agro-alimentary waste from Tommy Atkins mango pulp obtained from crop waste and different nitrogen sources; as well as its comparison with BC from pure carbon sources and its characterization in order to obtain cost-competitive and sustainable biomaterials, suitable for specific applications, such as biomedicine.

2 Materials and methods

2.1 MPW extraction and characterization

The mango variety used in this study was Tommy *Atkyns* coming from Amatitán, Jalisco, Mexico. The mango was processed to separate its pulp (MPW) by using of a 5 HP fruit-pulping machine (Alvarezmaq; MEXICO).

MPW was characterized in terms of: pH, reducing sugars (Miller´s DNS Method) and total sugars (Dubois' Phenol-Sulfuric method) on an UV-VIS Varian Cary 50 Spectrophotometer; specific carbohydrates analysis were carried out using a HPLC Waters equipped with a RI detector using an

Aminex HPX-87C column at 80°C, and water as the eluent, with a flow rate of 0.6 mL/min (20 μL of sample). The bromatological analysis was carried out following the applicable regulations: Ashes, NMX-F-607-NORMEX-2004; carbohydrates, NMX-Y-097-1974; raw fiber, NMX-Y-094-SCFI-2001; raw fat, NMX-F-615-NORMEX-2004; humidity, NOM-116-SSA1-1994; dry matter, NMX-F-083-1986; raw protein, NMX-Y-118-SCFI-2001.

2.2 Microorganism

Strain *Komagataeibacter xylinus* DSMZ 2004 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) and used to produce BC, it was propagated in solid HS medium (pH 5) (glucose, 20 g/L; peptone, 5 g/L; yeast extract, 5 g/L; dibasic sodium phosphate, 2.7 g/L; citric acid, 1.15 g/L; agar, 15 g/L) at 30 °C for 72 h.

The inoculum was prepared as follows: first a pre-culture was prepared in an Erlenmeyer flask with 50 mL of HS medium, inoculated with a loop of colonies grown in the solid HS medium. The flasks were incubated for 48 h at 150 rpm and 30 °C. This pre-culture was vigorously shaken with a vortex, and a volume of 100 μL (OD_{600}=0.1) was withdrawn and added to vials with 1 mL of HS medium. Vials were incubated for 48 h at 30 °C under static conditions. At the end of the incubation period, vials where shaken and 500 μL of it and 50% glycerol solution were added to the vials and frozen at -20 °C for future use as inoculum.

2.3 BC production from MPW

Culture media were prepared using MPW with a total sugars concentration of 50 g/L, and supplemented with different nitrogen sources: peptone (10 g/L); yeast extract (10 g/L); peptone/yeast extract (5:5 g/L); urea (2.4 g/L); ammonium sulfate (5.3 g/L), or without a nitrogen source. The concentrations of each compound were standardized so that the amount of nitrogen added was the same in all media. In half of the culture media, 2.7 g/L of Na_2HPO_4 and 1.15 g/L of citric acid were added as a buffer, considering that optimal growth of BC-producing strains is at pH 5-6.5. The different culture media (each one with three replicates) were heated for 7 minutes at 121 °C and 1 bar, prior to inoculation with *K. xylinus*. Control medium used was a modification of HS medium containing a mixture of sucrose, fructose and glucose in the proportion found in the mango (30 g/L sucrose, 15 g/L glucose, 5 g/L fructose) and peptone, 5 g/L; yeast extract, 5 g/L Na_2HPO_4, 2.7 g/L; citric acid, 1.15 g/L. The fermentation flasks used were 100 mL glass containers (10 cm diameter) with 20 mL of culture media, inoculated each with one *K. xylinus* culture vial (inoculum) described previously. After inoculation flasks were vigorously shaken with a vortex and incubated at 30°C in static conditions for 16 days.

At the end, the BC membrane was retrieved, washed and purified. Its wet weight, dry weight (without purification and purified) was quantified. Likewise, the pH of the remaining medium and the amount of final total sugars were measured.

Posteriorly a kinetic study of BC production was carried out for 28 days. Twenty-one culture flasks were filled with 20 mL of culture medium (which presented the best results regarding BC production), inoculated each with one *K. xylinus* culture vial as previously described and incubated statically at 30 °C. Three flasks were withdrawn at the following culture times (days): 0, 2, 5, 8, 12, 16 and 28, determining pH, BC and bacterial biomass (dry cell weight) produced (gravimetrically as mentioned further in the purification section), and sugar consumption (HPLC).

BC and bacterial biomass production curves (both reported as dry weight) were adjusted to the modified Gompertz equation (Hussein *et al*., 2017; Kim *et al*., 2018) (Eq. 1), using Matlab® software.

$$C = C_{\max} e^{-ae^{-\mu t}} \quad (1)$$

where $C_{\max}$ (g/L) is the maximum production (dry weight), corresponding to BC or bacterial biomass respectively; α it is a non-biological parameter related to the initial conditions of culture media; μ (1/d) is the rate of bacterial biomass or BC production and t (d) is the time of fermentation (Quintana, 2015; Winsor, 1932).

2.4 BC Purification and quantification

The BC membranes produced were withdrawn from the fermentation flask freeze-dried (dryer Freezone 4.5, Labconco, USA); at -45 °C and 0.8 MPa for 24 hours, and weighed (dry weight). Then they were purified as follows: washed with distilled water and immersed in a 0.1 M NaOH solution, heating at 90 °C for 30 minutes in a bath with controlled temperature. Subsequently, purified BC membranes were washed with distilled water until the pH of the wash water was 7, then freeze-dried as previously mentioned and weighed (dry weight).

The bacterial biomass was taken to be the difference between the weights of the dried unpurified BC and the dried purified BC (after treatment with NaOH) as reported by Jung *et al*., (2007).

2.5 BC characterization

BC membranes obtained were characterized by FTIR (Fourier transform infrared spectroscopy), XRD (X-ray diffraction), SEM (scanning electron microscopy), TGA (thermogravimetric analysis), and *WHC* (water holding capacity).

2.5.1 FTIR

The BC membranes infrared spectra were recorded in a Nicolet iS5 (Thermo Scientific, USA), equipped with an ATR (attenuated total reflection) device. The scan range was from 4000 to 400 cm^{-1}, using 32 scans and a resolution of 4 cm^{-1}.

2.5.2 XRD

An Empyream X-ray diffractometer (Malvern-Panalytical, UK) with a copper K_α (λ = 1.5406 Å) at 45 kV and 40 mA (2θ range from 5º to 50º with a step size of 0.02º s^{-1}) was used to analyze dried BC samples. BC crystallinity index (CrI^{XRD}) was calculated from the ratio of the height of the highest and the height of the minimum peaks with Eq. 2 (Park *et al*., 2003).

$$CrI^{XRD} = \left(\frac{I_{200} - I_{am}}{I_{200}}\right) \times 100 \quad (2)$$

where I_{200} is the maximum intensity of the (200) lattice diffraction and I_{am} is the intensity diffraction at 2θ (Shezad *et al*., 2010).

2.5.3 SEM

The BC membranes morphology was analyzed by scanning electron microscopy with a MIRA3 (TESCAN, Czech Republic). Dried thin BC membranes were coated with gold using an ion sputter coater. Fibers diameter were measured with Image Pro Plus® Software (50 measurements in 5 different regions).

2.5.4 TGA

Thermogravimetric analyzes were performed on a STA PT-1000 (Linseis, Germany) on BC samples (10 mg) at heating rate of 10 ºC/min from 25 to 500 ºC in air.

2.5.5 WHC

According to Zhong *et al*., (2013), humidity content was calculated measuring the wet weight of BC and subsequently, drying it in a vacuum oven on a Teflon plate for 10 hours at 80 °C and 0.8 MPa.

WHC was determined using the Eq. 3. For this parameter, it is important to stabilize the wet weight of the membranes, so BC membranes were dried 48 hours at ambient conditions of 27 ºC and 40 % relative humidity and then dried for 12 hours at 60 ºC in an oven. The results were obtained using Eq. 3 (Ul-Islam *et al*., 2012).

$$WHC = \frac{\text{Mass of water removed during drying (g)}}{\text{Dry weight of BC sample (g)}} \quad (3)$$

3 Results and discussion

3.1 MPW characterization

The bromatological analysis (Table 1) showed the content of nutrients in MPW, including protein, which is an important parameter to define if there is enough nitrogen to support the microbial growth or if it will be necessary to add an extra nitrogen source. It also provided data on the ash content. In this regard, Leterme *et al*., (2006) studied the mineral content of mango ash, reporting elements such as Ca, P, K, Mg, and Na that could favor the production of bacterial biomass and increase the yield of BC production.

Although, the percentage of crude protein is not equivalent to the nitrogen content in the mango pulp, it can give an approximation. This parameter is an estimate of the amount of nitrogen in the culture media that is available in the mango pulp. It can be inferred that the yield of the BC obtained in a mango-based medium could be lower without an external nitrogen source, since traditional BC medium contains an approximate amount of 1.1 g/L of nitrogen, mainly in the form of peptides and amino acids, present in peptone and yeast extract (Stanbury *et al*., 2017). The result of protein and ashes in MPW obtained in this work (Table 1) are lower than that reported by Surco-Laos *et al*., (2017), where the protein content was 1.04 % to 1.54 % and ash content was 0.58 % to 0.77 % for varieties of Venezuelan mango; however, they are similar to the percentages reported by Chauhan *et al*., (2001) where the protein content was 0.72 % and 0.46 % ash for a mango species from India.

Table 1. Results of the bromatological analysis corresponding to Tommy Atkins MPW used for culture media formulation.

Parameter	Percentage (% in W/W)
Ashes	0.37
Carbohydrates	10.65
Raw fiber	0.46
Raw fat	4.33
Humidity	83.87
Dry matter	16.13
Raw protein	0.32

As it is observed, the results of the bromatological analysis of mango may vary depending on the species of the fruit that is analyzed.

Sugars are the main carbon source for BC production therefore, it is very important to know the total amount and specific sugars contained in the mango-based medium. Total sugars amount in Tommy Atkins mango pulp was 186.1 ± 0.7 g/L, and reducing sugars concentration was 59.7 ± 2.8 g/L. The total sugar content reported by Liu *et al*., (2013) is slightly lower (154 g/L), while reducing sugars content (50 g/L) is similar. However, as mentioned before, the variety and maturation of mango are closely related with the change in its physical and chemical properties, such is the case described by Shaheen *et al*., (2015), who obtained only 103 g/L of total sugars, but the content of reducing sugars remained around 55 g/L.

Due to its high content of sugars, mango pulp has been used in biotechnological processes for the production of: bioethanol in batch fermentation (Somda *et al*., 2011) and by *Saccharomyces cerevisiae* and two more isolated strains of the coyol palm tree (Santiago-Urbina *et al*., 2011); in combination with pineapple juice and papaya puree for lactic acid production (Fessard *et al*., 2017); and vinegar production (Garg *et al*., 1995).

The results for the specific carbohydrates content in mango pulp were (%): sucrose, 59.9; glucose, 12.5; and fructose, 27.0 (these percentages were used to formulate the control culture media referred in this section). These results are very similar to those reported by Liu *et al*., (2014) where sucrose represented 69.7 %, fructose 20.5 % and glucose 9.9 % of the total sugars in culture media.

3.2 BC production from MPW

Fig. 1 shows the results obtained for the production of BC and bacterial biomass (both reported as dry weight) during 16 days of culture, varying the nitrogen source and the addition of buffer. As it can be seen, the media without any source of nitrogen had the lowest BC production (0.75 and 0.73 g/L) regardless de the use or not of buffer, due to the low amount of nitrogen contained in the mango pulp as previously mentioned. It was also observed that in all mango-based culture media, BC production was higher than that obtained in the control HS medium (1.94 g/L) which can be explained by the formation of D-gluconic and D-keto-gluconic acids in such medium, which decrease the optimum pH and with it, the cell viability (Keshk, 2014b). On the other hand, higher BC production in mango-based media could be due the presence of antioxidant compounds in mango, such as ascorbic acid (Liu *et al*., 2013) and beta carotene (Natal *et al*., 2017) which might reduce the formation of the organic acids (above mentioned) and improve the performance of BC production, as previously reported for culture media with other antioxidant compounds (Keshk and Sameshima, 2006).

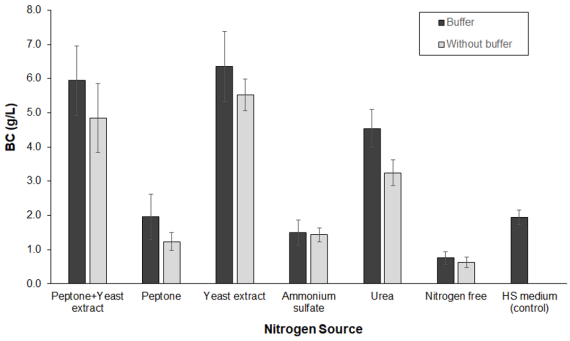

Fig. 1. Production of BC (g/L) from MPW after 16 days of static culture, varying nitrogen source and the addition or not of citrate buffer (30°C).

Culture media with buffer addition had a slightly higher BC production, reaching a maximum of 6.32 g/L (buffer addition and yeast extract as nitrogen source) versus 5.94 g/L (without buffer and yeast extract as nitrogen source). There are several explanations for cases in which BC production was higher with the addition of buffer. It has been reported that Acetobacter bacteria generate gluconic acid in the presence of glucose, whose accumulation or overflow reduce the pH of the culture media, affecting the yield of the fermentation (Zhang *et al*., 2016). The HS media prepared without Na_2HPO_4 presented a slight decrease in BC production. In this regard, Phosphorus is considered a critical nutrient for some bacterial growth processes (Stanbury *et al*., 2017). Its function in the formation of BC is directly linked to the

accumulation and release of energy in the metabolism of *K. xylinus* (Coyne, 2000), so it can be inferred that the lack of this nutrient causes a decrease in BC yield..

As previously mentioned, it was observed that the mango-based medium without additional nitrogen source presented the lower BC production (Fig. 1). This decreased production might be due to the low nitrogen content in mango pulp, as well as the lack of growth factors. On the other hand, complex nitrogen sources such as peptone and especially yeast extract contains growth factors, which stimulate bacterial growth. It has been reported that some amino acids or ions such as ammonium can stimulate or inhibit the growth of some *Acetobacter* bacteria (Álvarez-Cáliz *et al*., 2012), which gives meaning to the fact that the results shown in Fig. 1 are different despite having added the same amount of nitrogen in all cases. Amino acids such as glutamine, alanine, histidine and glutamic acid present in the peptone and yeast extract tend to increase the growth of the bacteria, while, in high concentrations, the ammonium ion present in the ammonium sulfate is an inhibitor of this type of cells (Sainz *et al*., 2017).

Regarding the effect of nitrogen source on BC production, Matsuoka *et al*., (1996) also found that BC production was higher in culture media with yeast extract and very low in absence of nitrogen. In more recent years, Sainz *et al*., (2017), worked on the addition of amino acids to the culture media, obtaining greater cell growth in those containing amino acids such as the present in the yeast extract, they concluded that single nitrogen sources were not able to support the growth of some strains as well as a complex solution of amino acids.

Mohammadkazemi *et al*., (2015) also studied the difference between three culture media using glucose as a carbon source and varying the nitrogen source, finding that when adding complex sources (corn liquor, peptone, peptone and yeast extract) BC production was higher specially in culture media containing a mixture of peptone and yeast extract. Based on the comparison of these authors results with those obtained from this work (6.36 g/L of BC with buffer and 5.94 g/L of BC without buffer), it can be concluded that it is important to add complex nitrogen sources to have greater BC production, and in this work, the selection of yeast extract as a nitrogen source for a maximized BC production. Fig. 2a shows the kinetic curves of BC production and biomass growth during 28 days of incubation using mango-based medium with yeast extract as nitrogen source.

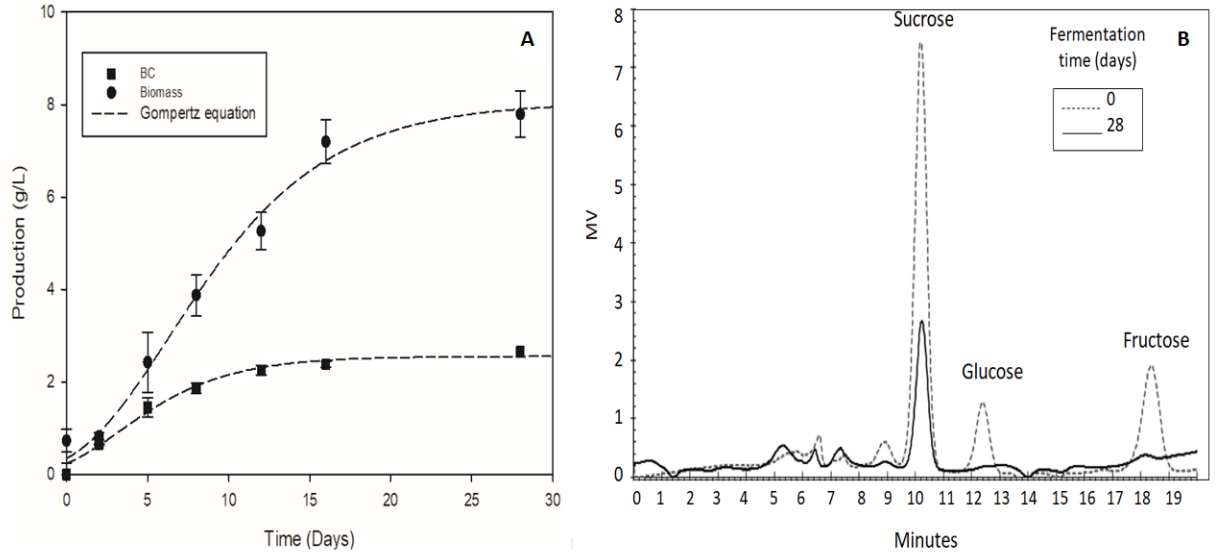

Fig. 2. Kinetics of BC, and Biomass production (a), in MPW-based culture medium in static culture using yeast extract as nitrogen source (initial pH 6, 30°C); Individual sugars consumption after 28 days of culture (b).

As shown in Fig. 2a, from day 0 to day 12 there is an exponential production of the BC, reaching its maximum at day 16, where the amount of BC obtained is around 2.4 g/L and maintains until day 28, where 2.6 g/L was the maximum production of BC. The pH dropped 1.5 units from day 0 to day 28 which might be related to the production of gluconic acids, as reported in other studies (Keshk, 2014b). BC production decreased although the biomass growth continues until day 16 of fermentation. In addition, this is probably caused by the oxygen depletion due that in initial stage, bacteria increase their population by taking oxygen dissolved in the medium and producing a certain amount of BC throughout the liquid phase. It has been established that BC production increases exponentially with respect to time when sufficient oxygen is available (Yamada *et al*., 2012). This behavior results in only the bacteria near to the surface being able to maintain their activity and produce BC (Krystynowicz *et al*., 2002).

Previously, a similar behavior to the observed in Fig. 2a has been reported, where about 75 % of BC production occurred exponentially in the first 9 days of incubation, and was reduced almost to a steady state until day 46 (Embuscado *et al*., 1994). In the same way, Lestari *et al*., (2014) reported the maximum biomass growth of *Acetobacter xylinum* in the logarithmic phase given in the first 36 hours of incubation, having an increase in BC yield in parallel with the bacterial growth, which was higher after 6 days. In addition, the general parameters of the fermentation kinetics in this study reveal a high rate of BC formation and efficient conversions of glucose to biomass and BC. Krystynowicz *et al*., (2002) reported that the yield of BC biosynthesis occurs with the increase in the number of cells and remains in the stationary phase until the sixth or seventh day, where the amount of production of BC is estimated at 0.5 g/L. It is important to consider in this case that the optimal conditions of aeration in culture media was determined, by the surface/volume ratio (S/V), and this is also an important factor that affects the synthesis of BC in static cultures.

Cell growth can be mathematically modeled to predict the population increase or the associated metabolite production. The Gompertz model arises from self-regulated growth models, where the growth rate decreases exponentially with time after reaching the inflection point (Hussein *et al*., 2017). The parameters shown in Table 2 were obtained fitting kinetics experimental data to Eq. 1.

Table 2. Kinetic parameters from the modified Gompertz equation.

Parameters	BC (dry weight)	Bacterial biomass (dry cell weight)
C_{max} (g/L)	2.56	8.06
α (-)	2.38	3.16
μ (1/d)	0.264	0.182
R^2	0.991	0.993

The parameter C_{max} indicates the maximum production of BC (2.56 g/L) and biomass growth (8.06 g/L) at day 28. As it is observed, a is very similar to the growth of the biomass and for the production of BC, because this parameter is associated to the conditions of the culture medium, which were the same for both kinetics. Specific growth rate, μ, is the biomass produced per unit of biomass and takes the unit per hours. In this case, the results were 0.264 1/d for BC production and 0.182 1/d for biomass. The difference between them can be related to the endogenous decrement due to cell death, or because the cells are using part of their energy for their own requirements, not only for the production of BC. Lestari *et al*., (2014) reports higher specific growth rates using culture media such as coconut water (0.619 1/d) but their maximum BC production (2.8 g/L) and biomass production (7.9 g/L) are similar to those obtained in this work.

Regarding sugar consumption, as shown in Fig. 2b, the microorganism completely consumed glucose (5 g/L) and fructose (15 g/L), while sucrose (30 g/L) was used in an approximately 60%, with remaining 12 g/L of this sugar in the medium at the end of the fermentation (28 days). Thus, 28 g/L of total sugars were consumed resulting in a $Y_{P/S}$ = 0.09, $Y_{X/S}$ = 0.29; and $Y_{P/X}$ = 0.31.

3.3 BC characterization

3.3.1 Morphological analysis by SEM

Morphological analysis of the BC shows the structure of its surface and allows for the identification of different characteristics to relate them with their intrinsic properties. Specific cases would be the porosity of the BC membrane and the dimensions of cellulose fibers formed. SEM characterization also showed the effect of the purification on the BC membranes. Fig. 3 shows SEM micrographs of the BC membranes in control and culture media with mango pulp after purification.

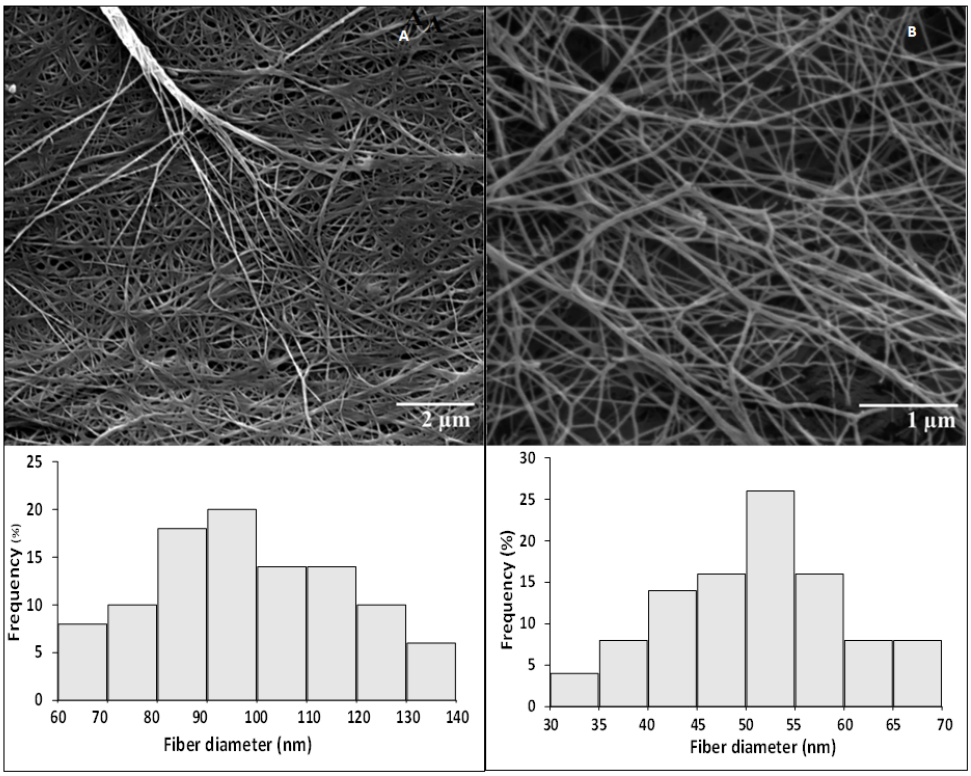

Fig. 3. SEM micrographs of the surface of purified membranes from BC after 16 days of fermentation produced in control culture medium (a); and MPW-based medium (b).

Both BC membranes show a compact cellulose network structure of a random assembly of microfibrils to form a uniform surface. In the two cases, the diameter of the nanofibers was less than 100 nm (98.76 ± 16.41 nm in control medium and 50.61 ± 13.7 nm for BC nanofibers obtained in mango medium). A smaller fiber size, as in the case of BC produced in mango medium, can provide a strong interfacial adhesion between the BC fibers and mayor superficial area (Mohammadkazemi *et al*., 2015).

After fermentation, BC contains remains of *K. xylinus* cells and components of the culture media (Chawla *et al*., 2009) which were removed via the treatment of BC membrane with 0.1 M NaOH. Fig. 3 shows the membrane free of biomass residues and with important reduction of impurities. As previously suggested, BC has medical applications, and although the purification does not guarantee an allergen-free material, it is an important step in the process to remove impurities, prior to the sterilization recommended for applications in this sector (Maneerung *et al*., 2008).

3.3.2 Crystallinity analysis by XRD

To measure the ratio between the crystalline (ordered) and amorphous (less ordered) regions of cellulose, a parameter called the crystallinity index has been used (CrI^{XRD}) which describes the relative amount of crystalline material in cellulose. Fig. 4 shows the diffractograms of the BC produced in control medium, and the BC obtained in mango-based medium. Each diffractogram has two diffraction dominant peaks, one between 14º and 15º, and other between 22º and 24º. Each of the peaks presents the two crystalline phases in cellulose, I_α and I_β (Santos *et al*., 2017). A decrease in peak intensity can be observed in BC from mango medium, compared with the control.

The average CrI^{XRD} is lower in the BC produced in mango medium (62.7 % and 77.2 % in control medium), however, both values remain above those reported for BC crystallinity (Santos *et al*., 2016). This difference may be due to the fact that complex culture media, such as mango pulp, contains Vitamin C, since it has been reported that low molecular weight compounds such as ascorbic acid have functional

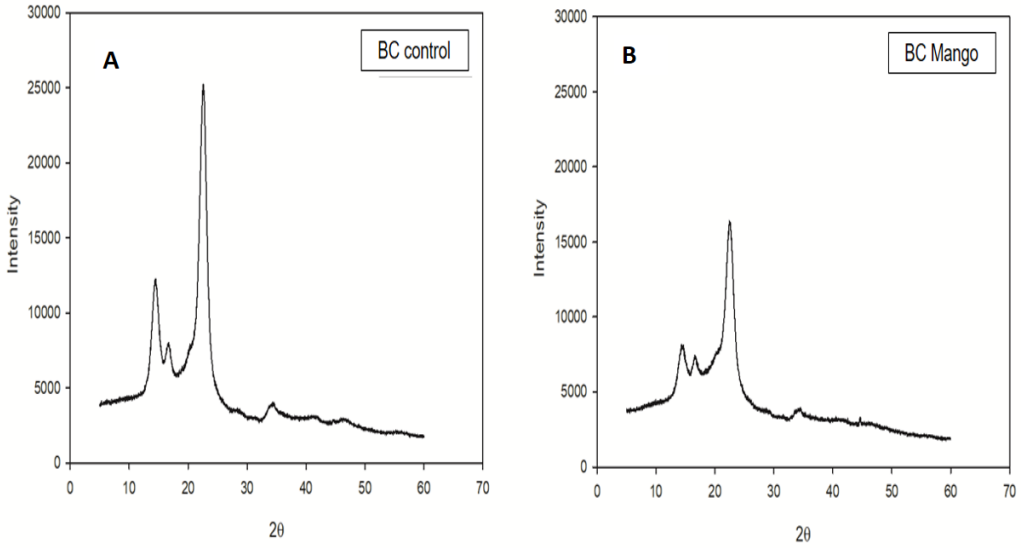

Fig. 4. X-ray dispersion plots (diffractograms) for the BC produced in control medium (a); and in MPW-medium (b), both were analyzed as powder.

groups capable of forming hydrogen bonds with cellulose, hindering the assembly of the microfibrils in cellulose fibers and decreasing the crystallinity (Keshk, 2014a). Mango pulp also contains significant amounts of polymeric substances such as pectin, which might interact with cellulose microfibrils assembly and decrease BC crystallinity, as observed in culture media with polymeric additives like xylans, that can cause decrement in crystallinity by inhibiting the aggregation of microfibrils during fermentation. Thus, these results in agreement with previous studies showing a reduction in crystallinity due to the presence of additives (Khan *et al*., 2015).

The lower crystallinity of BC from MPW (62.7 %) can increase the permeability to other reagents and water absorption of BC membrane and make it useful in cutaneous tissue regeneration applications (Roncero Vivero, 2001). For example, it has been studied that to improve the healing processes of diabetic foot ulcers, the wounds must be covered with biomaterials that protect against a possible infection, but at the same time avoid the dissection of the wound providing a humid environment that facilitates healing and oxygen permeability (Jannesari *et al*., 2011).

Crystallinity for BC produced in culture media with different carbon sources has been reported; in an HS medium with mannitol was 65.5 %; HS with sucrose, 63.2 %; Zhou medium with mannitol, 53.3%; and Zhou medium with sucrose, 46.7 %. In this work, the crystallinity for BC produced in the control (77.2%) was higher than the previously reported for similar culture medium, and crystallinity for BC obtained in mango medium (62.7%) is in the range of what has been obtained with conventional carbon sources.

3.3.3 Thermal behavior by TGA

Thermal behavior can provide ideas about BC fibers interactions, especially in cases that study the use a complex carbon source in the culture medium, due to interactions between BC and other fibers (Stumpf *et al*., 2013). Thermal degradation of the samples can be affected by different structural parameters, such as the molecular weight, the orientation of the fibers and the crystallinity of the BC (Urbina *et al*., 2017). Fig. 5 shows the thermograms for dried and powdered BC produced in control medium and mango medium.

BC from mango medium presents the same thermal behavior as that produced in the control. According to the thermogram, both samples show a weight loss of 10 % from 25 °C to 100 °C, mainly related to humidity of the sample, possibly due to both having the same moisture content. The behavior is stable up to approximately 225 °C, when the natural fiber degradation process begins and there is a weight loss of 60 - 70 %. The onset temperatures (T_{onset}) shows that the thermal behavior of BC was not substantially modified by the differences between both culture media (Stumpf *et al*., 2013).

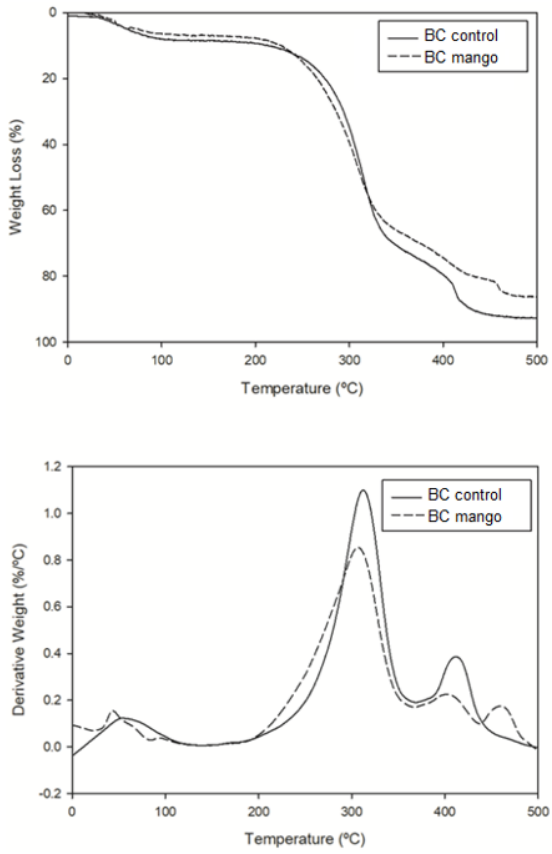

Fig. 5. TGA curves of powdered BC produced for the BC produced in control medium and in MPW-medium.

Pyrolysis of both BC start on a higher temperature range (340 - 360 °C) associated with the complete degradation of the BC, including the depolymerization, dehydration and decomposition of the glucose, similar to reported by Mohammadkazemi *et al*., (2015).

3.3.4 *FTIR-ATR spectroscopy*

Fig. 6 shows the FTIR spectra for BC produced in both media. Both show a very similar spectrum, indicating that the two samples have the same chemical structure typical of BC. All spectra shows the characteristic bands of cellulose, absorption bands near 3400 cm^{-1} and 700 cm^{-1} (Stumpf *et al*., 2013), corresponding to the -OH groups and peaks between 900 and 1200 cm^{-1} related to the structure of the glucopyranose rings (Shezad *et al*., 2010). Two bands in the FTIR spectra were founding the same range of wavenumber and did not show any significant difference.

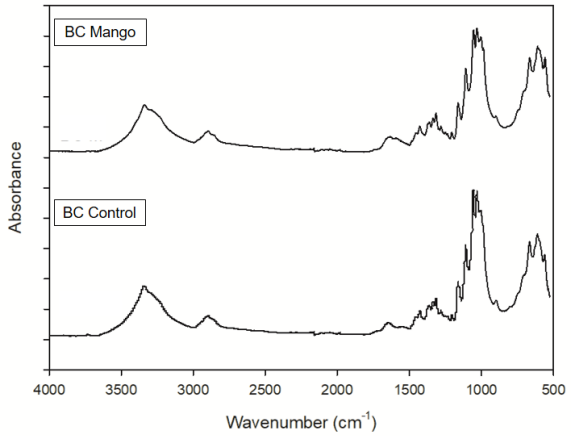

Fig. 6. FTIR-ATR spectra for BC membranes produced in control medium and in MPW-medium.

For vegetable cellulose sources may be present a peak at 1735 cm^{-1} corresponding to carbonyl groups of lignin (Yang *et al*., 2007), however, as is to be expected in BC samples, this does not occur, which confirms the purity of the material (Santos *et al*., 2016).

3.3.5 *Humidity content and WHC*

Average wet weight of BC membrane was 0.7010 g and dry weight was 0.0118 g. According to this result, 98.3 % of the membrane is water and 1.7 % corresponds to the weight of the BC.

Water holding capacity (*WHC*) was determined, which is an important parameter for BC with possible biomedical applications. This parameter expresses the amount of water that the BC can absorb with respect to the dry weight of the membrane. According to the results, the hydrophilic nature of the BC membranes produced in MPW-medium results in a WHC of 108.7 ± 3.3 times its dry weight, corresponding to the value reported by Ul-islam *et al*., (2012). This value was higher than the *WHC* of BC membranes produced in control medium (88.7 %). This parameter can be directly related to the porosity and the surface area of the BC membranes (Gelin *et al*., 2007).

There are different applications in the medical and pharmaceutical industry that require a high capacity for liquid retention. An example of this are the materials used for blood absorption (Harkins *et al*., 2014). Due to its biological characteristics, the BC could be used to facilitate cellular diffusion and proliferation as well as for the generation of tissue (Kirdponpattara *et al*., 2015). The high mechanical resistance of the membranes in the wet state and their

capacity for hydration and permeability of liquids and gases, in addition to the null or low irritation caused to the skin, make the BC membranes a useful material in the care and regeneration of cutaneous wounds (Keshk, 2014a) and a superior option to the use of autografts in patients with severe skin damage (Jozala *et al*., 2014).

Conclusions

The use of MPW for nanocellulose production by *K. xylinus* is feasible, obtaining a high yield; the process takes advantage of a highly generated agro-food waste in many countries and simultaneously, contributes to the reduction of production costs of BC. Some physical properties of mango-based BC were altered with respect to the obtained in a control media, such as crystallinity, *WHC*, and nanofibers diameter. Such differences could improve the BC water absorption and permeability to other reagents, which might be useful in the functionalization of the membranes for future biomedical and pharmaceutical applications.

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Nomenclature

BC	bacterial cellulose
OD_{600}	optical density of a sample measured at wavelength of 600 nm
C	concentration, g L^{-1}
e	Euler's number
t	time, d
C_{max}	maximum production, g L^{-1}
α	non-biological parameter related to the initial conditions of culture media, -
μ	rate of biomass or BC production, d^{-1}
CrI^{XRD}	crystallinity index, %
I_{200}	maximum intensity of the lattice diffraction, %
I_{am}	intensity diffraction at 2θ, %
WHC	water holding capacity, -
MPW	mango pulp
$Y_{P/S}$	product/substrate yield coefficient, -
$Y_{X/S}$	biomass/substrate yield coefficient, -
$Y_{P/X}$	product/biomass yield coefficient, -
T_{onset}	onset temperature

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