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Bacterial Cellulose from Simple and Low Cost Production Media by *Gluconacetobacter xylinus*

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Abstract Bacterial cellulose pellicles were produced by *Gluconacetobacter xylinus* using non conventional low-cost carbon sources, such as glycerol remaining from biodiesel production and grape bagasse, a residue of wine production. The carbon sources assayed showed their suitability for microbial cellulose production, with relatively high production values such as 10.0 g/l for the culture medium with glycerol from biodiesel as carbon source and corn steep liquor as nitrogen source; and 8.0 g/l for the culture medium containing grape bagasse and corn steep liquor. Glucose, commercial glycerol and cane molasses were also assayed as carbon sources for comparison. The bacterial celluloses produced were characterized by means of scanning electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy and thermogravimetric analysis. Morphological analysis showed that bacterial cellulose microfibrils produced from the non-conventional media used were several micrometers long and had rectangular cross-sections with widths and thicknesses in the range of 35–70 and 13–24 nm, respectively. X-ray patterns showed crystallinity levels in the range of 74–79 % (area method), whereas both X-ray patterns and infrared spectroscopy evidenced the presence of peaks characteristic of Cellulose I polymorph. Besides thermal properties were similar to those found for the pellicle obtained from glucose. The study performed showed the suitability of using wine residues or glycerol remaining from increasing biodiesel production as cheap carbon sources for production of bacterial cellulose microfibrils, with similar characteristics as those obtained by use of more expensive carbon sources such as glucose or commercial glycerol. On the other hand, the low cost nitrogen sources used (corn steep liquor or diammonium phosphate) also contributed to the economy of the bioprocess.

Keywords Bacterial cellulose · Low cost carbon sources · Grape bagasse · Glycerol from biodiesel

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Introduction

Cellulose is a linear polysaccharide consisting of a chain of β(1 → 4) linked D-glucose units. Although generally obtained from wood and natural fibers, it is now well established that cellulose is also produced by a family of sea animals called tunicates, several species of algae, and by some species of bacteria. In the last decade, nanosized constituents of cellulose have triggered a revived interest on the well-known natural polymer. During biosynthesis of cellulose chains, van der Waals forces and hydrogen bonding between hydroxyl groups and oxygens of adjacent molecules promote parallel stacking of multiple cellulose chains forming elementary fibrils that further aggregate into larger microfibrils [1], with diameters within 2–20 nm and several microns length.

In the last decade much research has been devoted to the extraction of cellulose microfibrils and nanocrystals from

wood and plant fibers by use of mechanical treatment or acid hydrolysis, respectively. A number of reviews on the extraction, properties and applications of microfibrillated cellulose and cellulose nanocrystals have been published in the last years [1–6]. On the other hand, microbial synthesis of cellulose in which specific bacteria synthesize cellulose microfibrils as a primary metabolite, appears as a promising route for the obtention of cellulose microfibrils. During bacterial cellulose (BC) synthesis, cellulose molecules are synthesized in the interior of the bacterial cell and spun out to form nanofibrils of ca. 2–4 nm in diameter which then aggregate in the form of ribbon-shaped microfibrils of ca. 80 × 4 nm [7]. The overlapping and intertwisted cellulose ribbons form a non-woven mat with very high water content. Bacteria-derived cellulose is of particular interest due to its light weight, high mechanical properties, non-toxicity, renewability, and biodegradability. Besides, the high chemical purity of the cellulose mat produced by bacteria avoids chemical treatments frequently used in plant-derived celluloses for the removal of hemicellulose and lignin.

BC is produced as an extracellular primary metabolite by bacteria belonging to the genera *Acetobacter*, *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Salmonella or Sarcina* [8, 9]. However, its most efficient producers are gram-negative acetic acid bacteria *Acetobacter xylinum* which has been reclassified and included within the novel genus *Gluconacetobacter,* as *G. xylinus* [10, 11]. Culture is carried out in static or agitated conditions at temperatures around 28–30 °C. Since bacteria used are aerobic, in static fermentations cellulose pellicle is formed only in the vicinity of the oxygen-rich air–liquid surface. The reason why the bacteria generate cellulose is unclear, but it has been suggested that it is a mean that bacteria use to maintain their position close to the surface of culture solution [12], as well as a protective coating that guard bacteria from ultraviolet radiation [13], or to prevent the entrance of enemies and heavy-metal ions whereas nutrients diffuse easility along the pellicle [7].

Although the first report on an extracellular gelatinous mat whose chemical composition and reactivity was the same as cell-wall cellulose dates from 1886 [14, 15], BC did not received much attention until the mid-1980s when remarkable mechanical properties of its pellicle were discovered [16, 17]. In the nineties the use of BC for composite materials was reported by several authors [18, 19], and in the following years the possibility of obtaining nanosized cellulose microfibrils of high purity with low energy input brought a resurgence in the area. Nowadays, proposed applications for bacterial cellulose include membranes for filtration, paper reinforcement, acoustic membranes, medicinal pads, hydraulic fracturing fluids for recovery of hydrocarbons, absorption composites such as nappies and sanitary products, reinforcement of polymer matrices, preparation of optically transparent films, tissue scaffolds, artificial skin bone cement, flexible displays screens, etc. [20–28].

In microbial fermentations, the cost of substrates normally accounts for up to 50–65 % of the total cost of production. BC production is not the exception, and thus in recent years much work has been devoted to find new low cost carbon sources. Recently, Castro et al. [29] produced BC microfibrils (2.8 g/l of medium) from non-conventional sources such as pineapple peel juice and sugarcane juice by use of a strain from *Gluconacetobacter swingsii* sp [29]. Moosavi-Nasab and Yousefi [30] studied the feasibility of using low quality date syrup—a fruit largely produced in the hot arid regions of Southwest Asia and North Africa—, for the production of BC using *G. xylinus*, obtaining a yield of 43.5 g/l of fermentation medium. To economize the BC production, Rani et al. [31] used coffee cherry husk extract (a byproduct from the coffee processing) and corn steep liquor (a byproduct from the starch processing industry) as less expensive sources of carbon and nitrogen, respectively, obtaining 6.24 g/l of BC in optimized conditions. Carreira et al. [32] studied the effect of several industrial residues (grape skins aqueous extract, cheese whey, crude glycerol and sulfite pulping liquor) on BC production with *G. sacchari*. They reported that productions can be substantially improved by the addition of N and/or P sources. They also reported that the responses in BC production were dependent on the raw material used, and also on the different N and P sources tested [32].

In the current work, production of bacterial cellulose by *G. xylinus* using as carbon sources glycerol remaining from biodiesel production and grape bagasse (a residue of white wine production in Mendoza (Argentina)) was studied, with the aim of formulating a general, simple and inexpensive medium to produce BC. More common carbon sources such as glucose, commercial glycerol and cane molasses were also assayed for comparison. Carbon and nitrogen sources used were compared not only in terms of cellulose yield and production, but also the morphology and structure of the BC obtained were studied. In this context, BC microfibrils produced in the selected media were characterized by means of scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and thermogravimetric analysis (TGA).

Materials and Methods

Microorganism

The bacterial strain of *G. xylinus* (*syn. Acetobacter aceti* subsp. *xylinus*, *A. xylinum*) NRRL B-42 used in this work

was gently provided by Dr. Luis Ielpi (Fundación Instituto Leloir, Buenos Aires, Argentina).

Cultivation Media and Conditions

Inocula were cultured for 48 h in Erlenmeyer's flasks containing Hestrin and Schramm (HS) medium (%, w/v): glucose, 2.0; peptone, 0.5; yeast extract, 0.5; disodium phosphate, 0.27; citric acid, 0.115. The pH was adjusted to 6.0 with dil. HCl or NaOH. Agitation (200 rpm) was provided by an orbital shaker. Culture media used for BC production were HS modified by replacing D-glucose by other carbon sources at the same concentration such as glycerol (commercial and that coming from biodiesel production) or cane molasses (50.75 % w/w fermentable sugars). Glycerol from biodiesel and grape bagasse alone or in the presence of 0.5 % w/v corn steep liquor (CSL) or 0.7 % w/v diammonium phosphate (DAP) as nitrogen source, were also assayed.

For BC production, static incubations were performed in Erlenmeyer's flasks for 14 days. The initial pH of the production media was 5.0. All cultures were incubated at 28 ± 1 °C and a ratio "volume flask: volume medium" of 5:1 was maintained. All media were sterilized by autoclaving (121 °C, 15 min).

To calculate cellulose production, pellicles were rinsed with water to remove the culture medium, and then boiled in 2 % w/v NaOH solution for 1 h in order to eliminate the bacterial cells from the cellulose matrix. Then, pellicles were washed with distilled water till neutralization. Dry weight was measured after drying the films at 37 °C till constant weight. Results were reported as "production" and expressed as gram dry weight of cellulose per litre of the original medium (g/l) [30]. Yield was calculated as gram dry weight of cellulose per gram of consumed substrate (g/g).

Pretreatment of glycerol remaining from biodiesel production was done as described by Chi et al. [33]. The product obtained (pretreated glycerol) contained 70 % (w/v) authentic glycerol. Glycerol concentration was determined enzymatically (Boehringer-Mannheim/R-Biopharm, AG, D-64293 Darmstadt, Ger.). Grape bagasse was homogenized in a blender with an appropriate volume of distilled water. The extract was then filtered through paper Whatman No. 3 and pH was adjusted to pH 5.0 with NaOH. Glucose and fructose contents were determined enzymatically (Boehringer-Mannheim/R-Biopharm, Cat. No. 10 139 106 035).

Conditioning of Bacterial Cellulose for Characterization Studies

For characterization studies the cellulose pellicles obtained were washed with water and homogenized in a blender for 5 min. The microfibrils thus obtained were treated for 14 h in a 5 % w/v KOH solution, rinsed with water till neutralization (pH = 7.0), and homogenized in water for 5 min [29]. For XRD, FTIR and TGA analysis, BC suspensions were dried on plastic Petri dishes at 45 °C during 3 h. For scanning electron microscopy the homogenate was conveniently diluted with distilled water.

Scanning Electron Microscopy (SEM)

Drops of BC/water suspensions were deposited on microscope glasses and dried at 45 °C for 3 h. Samples were coated with gold using an ion sputter coater, and observed by use of an scanning electron microscope Zeiss Supra 40 with field emission gun operated at 3 kV. Average diameter distributions of bacterial cellulose microfibrils were obtained from microscopic images by use of the ImageJ software, which measures the number of pixels in the picture and the scales lengths according to the calibration provided by the user.

X-ray Diffraction (XRD)

The structure of bacterial cellulose was analysed with a Bruker/Siemens automated wide-angle powder X-ray diffractometer. The X-ray diffraction pattern was recorded in a 2θ angle range of 0–40°. The wavelength of the Cu/Ka radiation source used was 0.154 nm, generated at accelerating voltage of 40 kV and a filament emission of 30 mA. X-ray diffraction data were analysed using MDI Jade 5.0 software. Curve-fitting was performed to find individual peak regions.

The crystallinity index (CI) of produced BC was determined by the following equation [34].

$$CI(\%) = \frac{I_c - I_{am}}{I_c} * 100 \quad (1)$$

where $I_{c\ or}$ I_{200} corresponds to the maximum intensity of the lattice diffraction, and I_{am} corresponds to the intensity of the peak at 2θ = 18°, which accounts for the amorphous part of cellulose. The intensity of the peaks was measured as the maximum value obtained for the peak taking into account a baseline.

Additionally, crystallinity of bacterial celluloses was determined by integration of each XRD peaks taking into account a baseline for each peak (area assigned to the crystalline part), and the total area under the diffractogram considering a straight line from 0 to 40° 2θ as baseline.

Fourier Transform Infrared Spectroscopy (FTIR)

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was performed using a Nicolet

6700 Thermo Scientific Spectrometer equipped with a Smart Orbit ATR accessory. The reflectance element was a diamond crystal. Spectra were collected in the 4,000–400 cm^{-1} range with 32 scans and at 4 cm^{-1} resolution.

Thermogravimetric Analysis (TGA)

Dynamic thermogravimetric analysis of dried samples was conducted in a TGA-50 Shimadzu instrument. Temperature programs were run from 25 to 550 °C at a heating rate of 10 °C/min, under nitrogen atmosphere (30 ml/min) in order to prevent thermoxidative degradation.

Results and Discussion

Bacterial Cellulose Production

As previously introduced, the cost of the carbon source plays a key role on bacterial cellulose production costs. In the current manuscript, low cost carbon sources such as glycerol obtained as byproduct of biodiesel production, and grape bagasse from regional wine production, were assayed. The volumetric productions (g/l) and the yield (g/g) of BC in HS culturing medium modified by replacing glucose by other carbon sources such as cane molasses (10.0 g/l of fermentable sugars) or glycerol (10.0 g/l of commercial grade or obtained from biodiesel production) are shown in Fig. 1. As it is indicated in the Figure, the highest production and yield were obtained for commercial glycerol. Results obtained agree with those of Keshk and Sameshima [35] who found a 155 % higher cellulose yield for BC production by *G. xylinus* in HS containing glycerol instead of glucose. Carreira et al. [32] reported higher productions of BC in the presence of analytical glycerol with respect to crude glycerol (2.07 and 0.10 g/l respectively) [32]. Kornmann et al. [36] also reported that glucose concentration had to be maintained at low levels during exopolysaccharides production by *G. xylinus*, in order to reduce the production of by-products as (keto)-gluconates that significantly diminished yields. The costs of carbon sources employed are also shown in Fig. 1, with the minimum found for glycerol obtained from biodiesel production. Although BC production using analytical grade glycerol was three times higher than production achieved using glycerol from biodiesel, production costs using the biodiesel byproduct could lead to values up to 18-folds lower.

BC production from a waste of white wine production such as grape bagasse (see Materials and Methods for details) containing 14.4–15.4 and 22.2 g/l fructose, was also assayed. The effect of the addition of compounds usually employed as nitrogen sources such as DAP and CSL was evaluated (Fig. 2). Results show that both productions and yields increased in the presence of DAP or CSL. Production values obtained were only slightly lower than in the presence of commercial glycerol as carbon source. Moreover, productions obtained with grape bagasse without supplementation were higher than those achieved with glucose or glycerol from biodiesel (see Fig. 1).

When experiments with glycerol obtained from biodiesel (20.0 g/l) alone or in the presence of DAP were carried out, pellicle production was not observed. In this sense, Carreira et al. [32] have also reported that $(NH_4)_2SO_4$ inhibited completely the cellulose production from crude glycerol by the bacterial strain *G. sacchari* isolated from a commercial food source [32]. However, when glycerol supplemented with CSL was used, the highest production was obtained, leading to values similar to commercial glycerol.

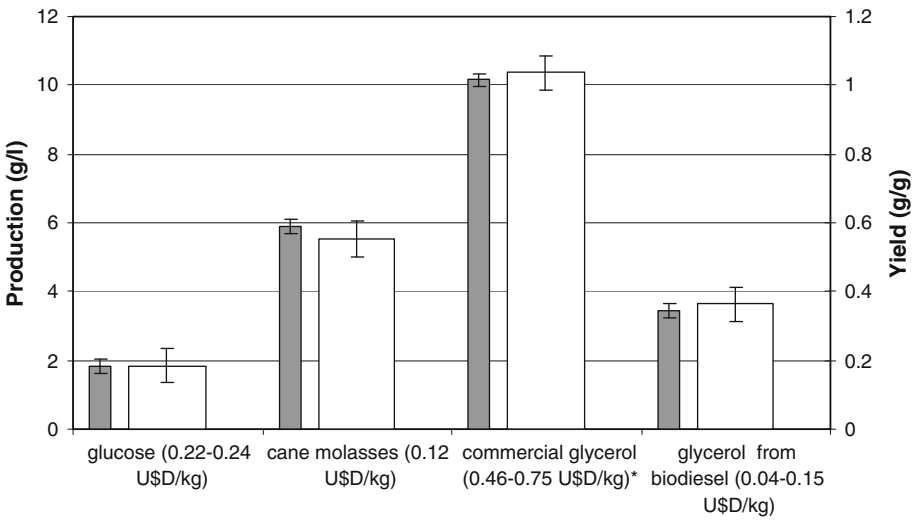

Fig. 1 Volumetric productions and yields of BC in the presence of different carbon sources: *filled square* Production (g/l); *open square* Yield (g/g). *Asterisk* Current international prices

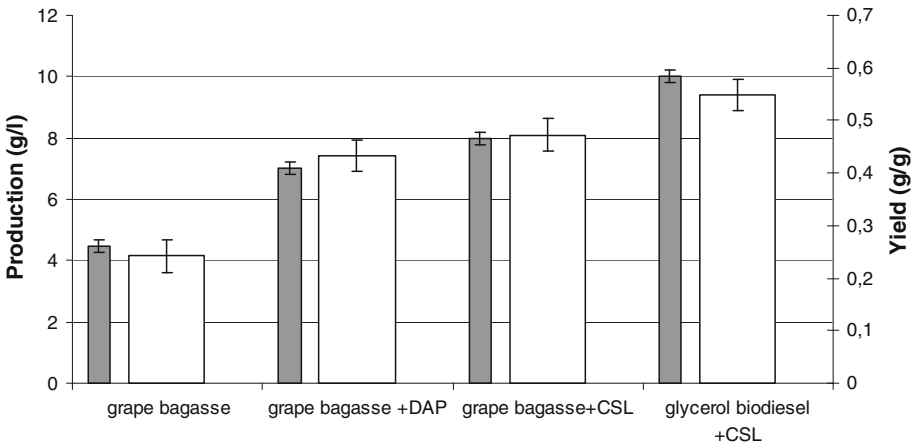

Fig. 2 Volumetric productions and yields of BC using as carbon source two agroindustrial wastes with and without supplementation: *filled square* Production (g/l); *open square* Yield (g/g). *CSL* corn steep liquor, *DAP* diammonium phosphate

Productivities achieved only with N supplementation (0.021–0.030 g/l h) largely encouraged the use of the non-conventional production media proposed, when comparing with the range of values reported in bibliography (0.009–0.018 g/(l h) [29, 31, 32]. In the case of grapes skins, Carreira et al. [32] found that the highest increment on BC production (nearly 85 %) was obtained when 4 g/l yeast extract and 2 g/l KH_2PO_4 were added as supplements. This condition corresponded to the maximum productivity of BC achieved by these authors (0.0104 g/l h) [32]. It is worth to notice that grape bagasse allows the production of an added-value product such as BC decreasing on the way environmental problems associated with its disposal, since only a very small portion of winery by-products are used world-wide [37]. On the other hand, as large quantities of glycerol are generated worldwide by the biodiesel industry and several other industries, its conversion to high-value added products is of great interest [38, 39]. The results obtained herein for both grape bagasse and glycerol from biodiesel are encouraging for the cost-effective industrial production of BC.

Scanning Electron Microscopy (SEM)

Figure 3a–f shows scanning electron microscopy images of BC obtained from the different carbon sources assayed. Ribbon like microfibrils forming a highly fibrous network-like structure can be seen (Fig. 3a). The bacterial strain of *G. xylinus* is also shown in Fig. 3a (see magnification square). Determination of microfibrils width distribution is not straightforward due to the frequent twisting of bacterial cellulose ribbons. Image analysis was then made manually in order to distinguish between widths, apparent thickness of edged on ribbons, and "intermediate" views. The analysis performed did not show the existence of significant differences among the dimensions of nanofibers obtained from the different carbon sources assayed, with nanofibers widths in the range of 35–70 nm, and thickness values in the 13–24 nm interval. Nanofibers widths are similar to those produced by other authors, whereas measured thicknesses are slightly higher [1, 29]. In terms of length, all microfibrils were several micrometers long since the microfibrils-ends were not seen even at low magnification (i.e. 5 K×).

X-ray Diffraction (XRD)

Cellulose has several crystalline polymorphisms (I, II, III, IV). Cellulose I is the crystalline cellulose that is naturally produced by a variety of organisms, i.e. trees, plants, tunicates, algae and bacteria. The structure of cellulose I is thermodynamically metastable and can be converted to cellulose II or III. All the cellulose strands are ordered in a highly ordered parallel arrangement [40]. The conversion from cellulose I to cellulose II can be produced by regeneration (solubilization and recrystallization) and mercerization (alkaline solution). Cellulose II has a monoclinic structure. Cellulose III is obtained from Cellulose I and II by means of liquid ammonia and thermal treatments. Cellulose IV is formed from Cellulose III [1].

One interesting characteristic of bacterial derived cellulose microfibrils is that it is possible to adjust culturing conditions in order to alter microfibril formation and crystallization [41, 42]. Diffraction patterns obtained for BC obtained from glucose, commercial glycerol, glycerol remaining from biodiesel production, grape bagasse and cane molasses are shown in Fig. 4. Three peaks shown in Fig. 4 at $2\theta = 14.62°$ (1–10), 16.29° (110) and 22.48° (200) (direction "c" of the unit axis is along the axis of the polymer for Miller indices) confirmed that only cellulose I was present in all BC samples [43–46]. No peaks, instead, are found at $2\theta = 12.1°$ and 20.8°, which are characteristic of cellulose II [47, 48]. Cellulose I is the crystal structure with the highest axial elastic modulus [1].

Crystallinity of cellulose can be determined by different methods, and the results are known to be dependant on the method applied [49]. However, results obtained by each

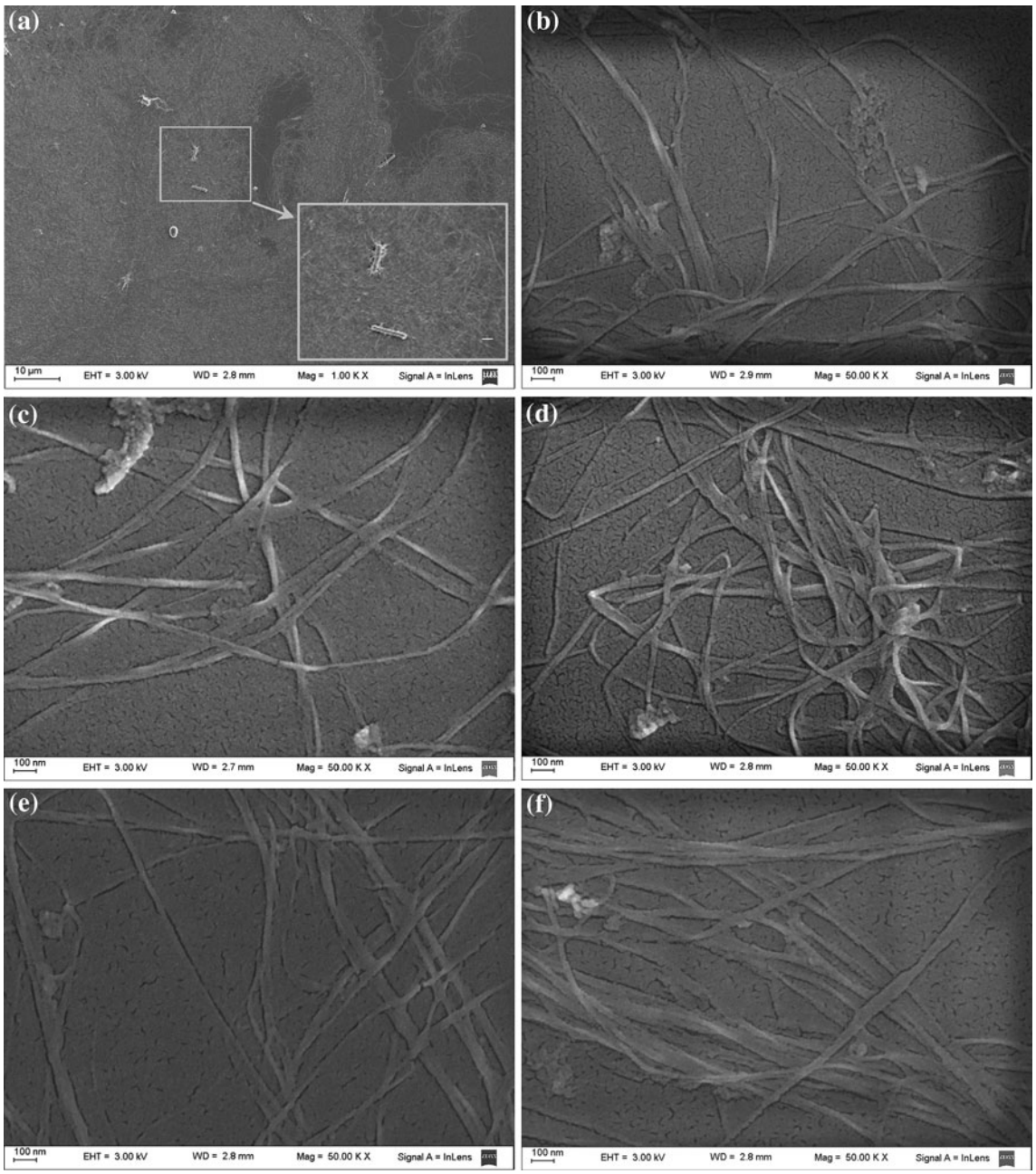

Fig. 3 (**a**) Bacterial strain of *G. xylinus* trapped in bacterial cellulose network (carbon source: grape bagasse). Bacterial cellulose from (**b**) glucose, (**c**) commercial glycerol, (**d**) glycerol remaining from biodiesel production, (**e**) grape bagasse, (**f**) cane molasses

particular method are useful for comparing samples identically analyzed. In the current manuscript, crystallinity was calculated by dividing the area of crystalline peaks by total diffractogram area (e.g. amorphous and crystalline zones). The areas were calculated using Origin software by fitting of multiple peaks, and by considering a connected baseline for each peak, and a straight line from $2\theta = 0°$ to $2\theta = 40°$ for the total area (see example in Fig. 5).

It is worth noting that the method described is undoubtedly influenced by the baselines that the operator takes into account, both at the base of each peak and also for the whole diffractogram. Calculated crystallinity values are shown in the first results column of Table 1. Results show that bacterial celluloses obtained from wine production residues or from the glycerol remaining from biodiesel production, have similar crystallinity values than those obtained from commercial glycerol or glucose (i.e. 74–79 %). The use of cane molasses as carbon source apparently leads to cellulose microfibrils with lower crystallinity (67 %).

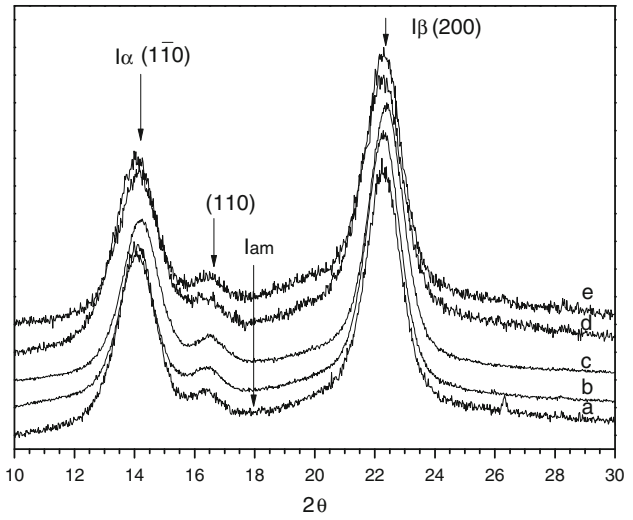

Fig. 4 XRD patterns of BC obtained from different cultivation medium. Patterns were vertically moved for a clearer view of each diffractogram. (**a**) glucose, (**b**) commercial glycerol, (**c**) glycerol remaining from biodiesel production, (**d**) grape bagasse, (**e**) cane molasses. Diffraction patterns have been normalized with respect to the peak at 2θ = 22.48°

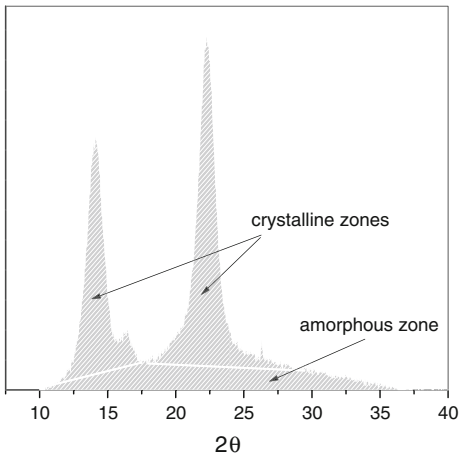

Fig. 5 Method used for determining the crystallinity based on crystalline peaks and total pattern area comparison. The area of the peak at 2θ = 16.29° was included in the area below the peak at 2θ = 14.62°

Besides, the crystallinity index (CI %) of BC samples was calculated as described in Materials an Methods after subtraction of the background signal measured without cellulose using the XRD patterns [34]. The I_{200} intensity was measured from the top of the 22.48° peak to the straight baseline of the diffractogram, and I_{am} was measured at 18° as a representative of the amorphous cellulose fraction. Results have been included in Table 1. Despite the method used provides a simple tool to measure crystallinity, there are some issues that should be taken into account: (a) the measure of the height of the peak at 18° as the amorphous part is underestimated because the maximum of the amorphous cellulose part was found to be shifted to higher angle [49]; (b) the measurement of the height of the peak without taking into account its area is an oversimplification of the determination; (c) the method considered only the highest peak (I_{200}) which accounts for the aligned cellulose crystal lattice, without taking into account the others peaks. Table 1 shows that determination of CI with the method proposed led to higher crystallinity values than the area method. On the other hand, both set of crystallinity determinations evidence that results obtained for different carbon sources assayed are very similar. However, it seems that cane molasses cultivating medium produces a bacterial cellulose with a slightly lower crystallinity than the others carbon sources. The behaviour could be ascribed to the glucan chain hydroxyl group are not allowing to self-assembly and there is a reduction in the crystallinity.

It has been reported that cellulose I structure is made of parallel chains characterized by an intermolecular hydrogen bond network extending from the O_2–H hydroxyl to the O_6 ring oxygen of the next unit [50]. Cellulose I has two polymorphs: a triclinic structure (Iα) and a monoclinic structure (Iβ), which coexist in various proportions depending on the cellulose source [2, 51, 52]. The Iα unit cell contains one chain, whereas cellulose the Iβ unit cell contains two parallel chains [53]. The ratio between Iα and Iβ polymorphs depends on the source of the nanocellulose [54]. However it is controversial due to the difficulties in structural characterization of individual nanocellulose [35]. Moon et al. [1] and Keshk and Sameshima [35], showed that Iα/Iβ ratio can be modified by culturing conditions (stirring, temperature, and additives). The presence of additives interferes with the aggregation of the elementary fibrils into the normal ribbon assembly, and chemically addition media may result in increased contents of Iβ crystal structure [42]. Iβ polymorph is preferentially

Table 1 Crystallinity, crystallinity index (CI) and Iβ content of BC obtained by use of different carbon sources

Carbon source	Crystallinity (%)[a]	CI (%)[b]	Iβ/Iα	Iβ (%)[c]
Glucose	77	92	1.45	31
Commercial glycerol	79	95	1.79	44
Glycerol from biodiesel	76	94	1.73	42
Grape bagasse	74	89	1.39	28
Cane molasses	67	89	1.39	29

[a] Crystallinity (%) was calculated as the ratio of the crystalline peaks and total diffractogram areas (Fig. 5)

[b] Crystallinity index, CI (%) was calculated from the maximum intensity of the lattice diffraction and the intensity at 2θ = 18°

[c] Iβ % was calculated as (Iβ − Iα)/Iβ × 100 where I is the height of the peak taking into account a baseline at the peak

formed in the isolated elementary fibrils that are free from constraint present when aggregated in the normal microfibril ribbon assembly. It has been suggested that this added constraint is necessary for the formation of the metastable Iα phase [55]. The ratio between both polymorphisms affects the inter-hydrogen bonding and may affect mechanical properties of BC. Moreover, the Iα/Iβ could also alter the width of the microfibrils leading to square instead of rectangular cross-sections of the BC ribbons [1]. Table 1 shows Iα/Iβ and the percent fraction of Iβ polymorph in BC calculated as $(I\beta - I\alpha)/I\beta$. Results show similar contents of the Iβ polymorph for glucose, cane molasses and grape bagasse carbon sources (28–31 %). On the other hand, commercial glycerol and glycerol from biodiesel production showed increased contents of the Iβ polymorph with values in the 42–44 % range. Changes in Iα/Iβ in the range found did not produce changes in ribbons widths sections that could be distinguished from the SEM images obtained.

Fourier Transform Infrared Spectroscopy (FTIR)

Figure 6 shows the FTIR spectra collected for the bacterial celluloses produced by use of the different carbon sources assayed. Spectra showed bands typical of cellulose I. The band centered at 896 cm^{-1} is typical of β-linked glucose polymers [29]. The band at 1,050 cm^{-1} could be associated with ether C–O–C functionalities [30]. The band at 1,109 cm^{-1} is assigned to C–O bond stretching [29]. The band at 1,159 cm^{-1} is assigned to cellulose C–O–C bridges [31]. The weak band found at 1,211, 1,275, 1,314 and 1,334 cm^{-1}, can be ascribed to O–H in plane vibration, C–H bending, CH_2 wagging, and O–H in-plane bending,

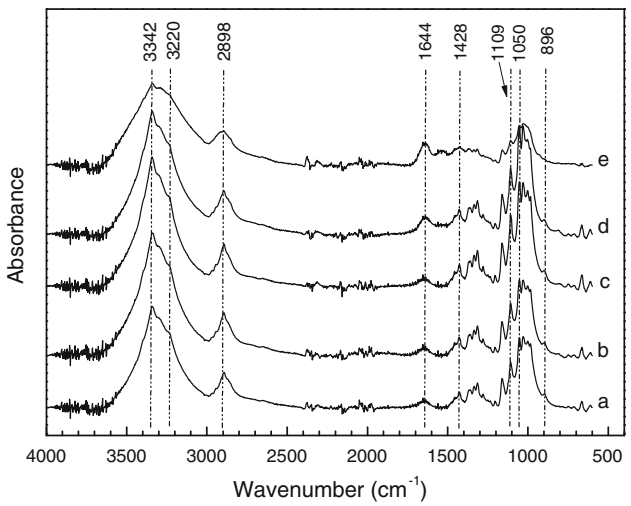

Fig. 6 FTIR-ATR of BC from different culture medium: (**a**) glucose, (**b**) commercial glycerol, (**c**) glycerol remaining from biodiesel production, (**d**) grape bagasse, (**e**) cane molasses

respectively [29]. The band centered at around 1,428 cm^{-1} could be associated with either CH_2 symmetrical bending or surface carboxylate groups. The band at 1,644 cm^{-1} is due to the H–O–H bending vibration of absorbed water molecules [31, 56]. The band at 2,898 cm^{-1} is attributed to CH_2 stretching. The bands at 3,220–3,342 cm^{-1} indicate inter-molecular and intramolecular hydrogen bonds [43]. Hydroxyl groups (–OH) appear at around 3,350 cm^{-1}. However, the analysis of the zone from 3,000 to 3,600 cm^{-1} is not very clear. The bands centered at around 3,220 cm^{-1} and at 750 cm^{-1} have been reported to account for the triclinic Iα allomorph, whereas bands centered at around 3,283 cm^{-1} and at 710 cm^{-1} are said to belong to the monoclinic Iβ allomorph [57]. Sugiyama et al. [58] worked with different modified BC and they said that the shrink or disappearance of 3,240 cm^{-1} peak in the spectrum implies a lower Iα content, and indicates that the inter-molecular hydrogen bonds are weaker and crystallinity content is low. However, the increasing of the amorphous part influences the hydration ability of bacterial cellulose, and as consequence it could also produce a higher OH content and deformation of this big peak. As it is shown in Table 1, from our results the BC from cane molasses has the lower intensity peak with a greater number of exposed free OH groups as compared with the other BC. Iβ peaks at 710 cm^{-1} in BC from cane molasses agree with its low crystallinity. The grape bagasse derived BC did not show the same behavior, which may be due to the higher water absorption of the sample in the air during the experiment. The amorphous part of the specimen also can absorb more water from the atmosphere. Park et al. [49] said that this method is influenced by the amorphous and crystalline regions.

Thermogravimetric Analysis (TGA)

Figure 7 shows the TG curves of the pellicles obtained for the different carbon sources assayed, in terms of percent weight of the original sample versus temperature. In all the samples two significant mass losses are observed. The first one takes place from room temperature to 150 °C and it is assigned to membrane dehydration. Physically adsorbed and hydrogen bond linked water molecules can be lost at that first stage [59]. The second mass loss is observed from 200 to 400 °C, and is assigned to thermal decomposition of cellulose [59].

Table 2 summarizes data on the onset degradation temperature, the temperature of maximum weight loss rate, and the pyrolysis residue remaining after heating the samples up to 550 °C. The extrapolated onset temperature (T_{onset})—which denotes the temperature at which the weight loss begins—was taken as the temperature in which the mass loss started, as it is shown in Fig. 7. The temperature of maximum weight loss rate (T_{max}) was calculated from the first

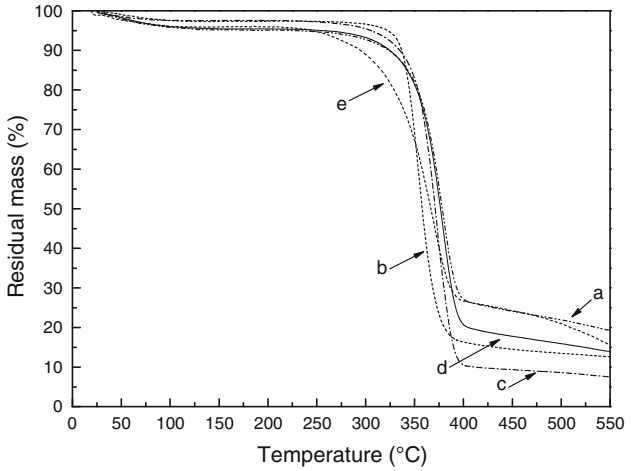

Fig. 7 TG curves of BC from different culture medium: (**a**) glucose, (**b**) commercial glycerol, (**c**) glycerol remaining from biodiesel production, (**d**) grape bagasse, (**e**) cane molasses

derivative of the TG data, and it indicates the point of greatest rate of change on the weight loss curve.

Results obtained for the non-conventional carbon sources assayed are similar to those found for glucose derived pellicles (Table 2). On the other hand, cane molasses BC showed T_{onset} and T_{max} values lower than the other BC pellicles. Thermal degradation behaviour is known to be affected by some structure parameters such as molecular weight, crystallinity and orientation of the fibres [34, 59]. In the case of cane molasses BC, XRD results showed that it had the lowest crystallinity (Table 1), which could be the reason for thermal behaviour observed. In the case of the mass loss profile of BC obtained from analytical glycerol, the sharp decrease in weight evidenced for this sample could be adscribed to its high crystallinity (Table 1). The maximum decomposition temperature is a criterion of thermal stability. BC from glucose has one of the highest values of T_{max} which indicates its higher thermal stability when compared with cane molasses and analytical glycerol BC. Glucose pellicle has the highest carbon residue content as well. Glycerol from biodiesel and grape bagasse carbon sources led to BC pellicles with T_{max} values similar to glucose BC.

Table 2 Onset degradation temperature, maximum weight loss rate temperature, and pyrolysis residue of BC pellicles at 550 °C (%)

Carbon source	T_{onset} (°C)	T_{max} (°C)	Pyrolisis residue at 550°C (%)
Glucose	221	378	19
Commercial glycerol	235	353	13
Glycerol from biodiesel	218	373	8
Grape bagasse	230	379	14
Cane molasses	206	368	16

Conclusions

Carbon source price plays a key role on the costs of industrial BC production. Aiming to reduce BC costs, low-priced carbon sources such as glycerol obtained as byproduct of biodiesel production, and grape bagasse from regional wine production, were assayed. Both, biodiesel glycerol and wine residues showed their suitability for high BC production (10.0 and 8.0 g/l, respectively) when supplemented with corn steep liquor for nitrogen requirements. Production values obtained were much higher than those achieved with HS culture medium, and similar to the ones found with HS culture medium modified by replacing D-glucose by commercial glycerol. With respect to nitrogen sources added (DAP or CSL), they also contributed to the economy of the bioprocess, as high values of productivity were obtained without the use of more expensive raw materials such as yeast extract.

BC ribbons obtained in non-conventional culture media showed widths values in the range of 35–70 nm and thicknesses values in the 13–24 nm interval, which were similar to cross-sections dimensions of ribbons found for BC obtained from glucose or commercial glycerol media. X-rays patterns and FTIR spectra confirmed the presence of cellulose I crystalline polymorphism. Crystallinity and crystallinity index values determined for bacterial celluloses obtained from wine production residues and from the glycerol remaining from biodiesel, were similar to those obtained for commercial glycerol or glucose BC. Morphological, structural and thermal similarities between microbial cellulose microfibrils derived from the low-priced media assayed and those obtained from traditional culture media (i.e. glucose), as well as the high production values achieved, suggest that grape bagasse and abundant glycerol remaining from increasing biodiesel production are attractive carbon sources for reducing the costs of industrial bacterial cellulose production.

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