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Superabsorbent bacterial cellulose spheres biosynthesized from winery by-products as natural carriers for fertilizers

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ABSTRACT

Soil contamination, sustainable management of water resources and controlled release of agrochemicals are the main challenges of modern agriculture. In this work, the synthesis of sphere-like bacterial cellulose (BC) using agitated culture conditions and *Komagateibacter medellinensis* bacterial strain ID13488 was optimized and characterized from grape pomace (GP). First, a comparative study was carried out between agitated and static cultures using different nitrogen sources and applying alternative GP treatments. Agitation of the cultures resulted in higher BC production yield compared to static culture conditions. Additionally, Water holding capacity (WHC) assays evidenced the superabsorbent nature of the BC biopolymer, being positively influenced by the spherical shape as it was observed an increase of 60% in contrast to the results obtained for the BC membranes under static culture conditions. Moreover, it was found that sphere-like BCs were capable of retaining urea up to 375% of their dry weight, rapidly releasing the fertilizer in the presence of water. According to our findings, sphere-like BCs represent suitable systems with great potential for actual agricultural hazards and grape pomace valorisation.

1. Introduction

In modern society, as a great example of constant expansion, proper management of resources and wastes is mandatory. Taking into account the dependence on petroleum and its derivatives, circular economy is gaining relevance as an option of sustainable development. In this context, biobased materials synthesized from agro-industrial wastes are a good example of sustainable material engineering.

Grape Pomace (GP), also called grape marc, is one of the most abundant agro-industrial wastes around the world and it is composed of the remaining mixture of skins, stalks and seeds from grape pressing in wine industry. The annual grape production worldwide is around 78 metric tons and the 57% is destined to wine making [1]. It is estimated that 20-25% of grape production ends up in GP [2]. As a result, millions of tons of GP are generated every year and due to its low pH and the high polyphenol content, the accumulation of this by-product could be polluting for the environment, being able to inhibit germination when it is untreated [3,4]. Particularly, in the case of white grape winemaking, GP retains higher amount of polyphenols in comparison with red grapes due to differences in maceration and fermentation processes [5]. Therefore, white GP needs to be managed even more carefully given this added potential risk to the environment. Hence, several strategies for valorisation of these residues have been described up to now.

GP has been utilized for bioenergy production and for butanol and biogas generation, but the above mentioned large amount of polyphenols and fermentable sugars makes GP a by-product with great potential for numerous other applications as strong liquor, cosmetic, pharmaceutical or food industries [6–8]. Actually, GP has been used as a carbon source in bacterial culture media for the production of lactic acid and bacterial cellulose (BC) [9–11].

BC is synthetized extracellularly by some strictly aerobic Gram-negative bacterial strains, showing greater purity, higher crystallinity and better mechanical properties compared to plant cellulose [12]. In static culture conditions, BC is produced in form of a microfibrillated pellicle in the air-liquid interphase. It has been reported that BC works as a protecting barrier for growing bacteria from ultraviolet radiation, other competitors and heavy-metal ions [13]. This BC membrane allows the exchange of nutrients while maintains bacteria close to air, thus ensuring the oxygen supply. Acetic acid bacteria can grow and produce BC with high purity and crystallinity from different agro-industrial by-

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products and fruit residues. Therefore, the number of studies focused on cost-effective carbon feedstocks has increased in recent years [14].

On the other hand, in agitated cultures BC grows in form of individual and nearly spherical groups, and both the composition of the medium and the agitation conditions influence the nucleation processes and the final shape of BC [15]. It has been previously reported that the agitation speed could be used in order to optimize the synthesis of BC in the form of asterisks, spheres or filaments [16]. Likewise, physicochemical characteristics of BC depend on the production mechanism, thus causing the potential application of the biopolymer to vary when cultured in agitated conditions. As a result, whereas BC in membrane form has been used in food industry, regenerative medicine, active composites and electronics [17], BC spheres have been reported with enhanced adsorption capacity or enzyme immobilization capabilities in material science and biomedical areas [18]. Nevertheless, little is known about the morphology and properties of BC synthetized from agricultural residues under agitated conditions and their possible effects on the nucleation processes in BC. Hence, there is a strong need for studies that exploit the great advantages of BC spheres in a large number of advanced fields, by exploring the main factors that control their formation.

Apart from residue accumulation, other challenging agricultural concerns are the over usage of agrochemicals for crop growth or protection and the fresh water exploitation [19,20]. The development of controlled release systems and superabsorbent hydrogels is an alternative to these threats [21,22]. In this way, it is intended to prevent the excessive application of harmful pesticides from contaminating food or water bodies. More recent research works promote the use of biodegradable polymers such as alginate, cyclodextrins, chitosan or cellulose and their derivatives, that have the ability to attenuate or control the release of agrochemicals, so that it continues to reach the target [23–25]. The common characteristic of natural polymer-based hydrogels is their ability to retain water, given their three-dimensional macromolecular structure, which is also beneficial for agriculture when crops are subjected to abiotic stress conditions such as water shortage [24]. Thus, cellulose-derived superabsorbent polymers have already been proposed since they offer similar performance in water optimization to acrylate-based superabsorbent polymers, and are environmentally friendly [26].

This research focuses on the production and characterization of BC with sphere-like morphology from GP agricultural residue. *Komagateibacter medellinensis* bacterial strain ID13488 was chosen since it can produce high yields of BC from low cost carbon sources and acidic culture media [27]. Due to the acidity of some agro-industrial residues and fermentation environments, *K. medellinensis* could prevent previous pH neutralization steps of the raw material.

In this way, the main aim was to provide insight into the biosynthesis process of BC spheres through the revaluation of GP agro-industrial residue. Therefore, the growth of K. medellinensis was studied under both static and agitated culture conditions. As a result, the development of sphere-like BCs biosynthesized from GP culture media was optimized. Finally, in order to expand BC applicability, the superabsorbent nature of the sphere-like BCs and their urea retention capacity were assessed.

2. Materials and methods

2.1. Materials and reagents

D-Glucose ($C_6H_{12}O_6 \geq 99.0\%$), yeast extract (≥89.0%), disodium hydrogen phosphate ($Na_2HPO_4 \geq 99.0\%$), citric acid ($C_6H_8O_7 \geq 99.5\%$) and 4-(dimethylamino)benzaldehyde 99% were purchased from Sigma Aldrich and peptone (≥85%), potassium hydroxide (KOH) and hydrochloric acid (HCl 37%) were obtained from Panreac Applychem (Spain). Acetonitrile was acquired from Scharlab (Spain). GP culture medium was prepared with *Hondarrabi zuri* white grape variety pomace, which was kindly provided by Bodega Butroi, a local winery (Biscay, Spain).

Following the criteria of an oenologist, the grapes were harvested at their optimum point of ripeness, according to the potential alcohol level after the winemaking process and the acidity index of the grapes. GP was collected the same day of grape harvest, after destemming and pressing the grapes under identical conditions. Finally, GP consisting on some stems, sheds and skins, was stored at −80 °C until its analysis.

Komagataeibacter medellinensis bacteria strain ID13488 (CECT 8140) was isolated from vinegar broth fermentation and kindly supplied by New Materials Research Group, Pontificia Bolivariana University of Medellín, Colombia [28].

2.2. Bacterial cellulose biosynthesis

BC membranes and sphere-like BCs were biosynthesized by *K. medellinensis* in white grape pomace culture media, both under static and agitated growth conditions.

For the preparation of the GP culture medium, first GP was crushed with a blender and different mixtures with ratios ranging from 2.5% (*w/v*) to 20% (w/v) of residue/water were prepared. The GP mixtures were passed through a cloth strainer and the filtrated was subjected to two types of treatments, ultrasonication and hot water hydrolysis, either individually or in combination. These treatments were used to facilitate the accessibility and diffusion of nutrients in the medium, the extraction of fermentable sugars and to reduce the amount of suspended solids. The ultrasonication was conducted for 30 min at room temperature (JP Selecta 3,000,683 50/60 Hz), whereas the hot water treatment was carried out at 80 °C for 1 h. Subsequently, the mixtures were centrifuged at 4500 rpm for 45 min (Hettich Zentrifugen D-78532). The pH of the solutions was then adjusted to 3.5 with citric acid if necessary and autoclaved separately at 120 °C for 15 min before inoculating the bacteria. The inoculum of a commercial HS medium in the exponential growth phase was stablished as 1% (*v*/v) for static cultures and 1.5% (v/v) for agitated cultures. 150 mL and 100 mL volumes of culture medium were used in 250 mL Erlenmeyer flasks for static and agitated growing conditions respectively. Finally, GP juice was mixed with peptone or yeast extract to analyse the effect of each supplement on the culture medium. Culture media with additional nitrogen sources were labelled with *pp* and *ex* in reference to peptone and yeast extract, respectively. The agitated cultures were subjected to rotatory speeds of 120 rpm, 130 rpm and 150 rpm in an IKA KS 4000i Control (Germany). All culture media, both under static and agitated conditions, were incubated at 28 °C for 7 days.

Lastly, obtained BC samples were washed with KOH at 2% (*w/v*) for 24 h to remove all non-cellulosic components and afterwards, they were subjected to several washes with deionized water until total neutralization. Finally, BC samples were dried in the oven at 50 °C until constant weight or freeze-dried (Telstar LyoQuest HT40) for their subsequent characterization.

Hestrin-Scharmm (HS) medium was used to stablish standard growth conditions: 2% (*w/v*) D-glucose, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.27% (w/v) disodium phosphate (Na_2HPO_4) and citric acid to adjust pH to 3.5 [29]. HS culture media were subjected to the same agitation, incubation and washing conditions as explained before for the GP media.

2.3. Characterization of culture media

2.3.1. BC yield

In both growing conditions, BC production was calculated after 7 days of incubation to better compare the yields following Eq. (1). Values were taken in triplicate.

$$Yield\left(\frac{g}{L}\right) = \frac{m_d}{V} \quad (1)$$

where m_d is the dried BC mass in grams and V is the total volume of the culture media in litres.

2.3.2. Culture composition

In order to characterize GP mixtures and the effectiveness of extraction treatments, the amount of D-glucose and D-fructose in treated and untreated preparations was analysed by spectrophotometry. Measurements were carried out before nitrogen supplementation, using a commercial kit from Byosystems (Spain). The content of these reducing sugars was followed measuring the absorbance change of the reduction of NADPH at 340 nm in the presence of phosphoglucose isomerase by a Shimadzu UV–Vis-Nir 3600 spectrophotometer (Japan).

To appreciate the in-depth behaviour of the bacteria in the culture medium, detailed sugar analysis was conducted during BC biosynthesis process. For that, the content of glucose, sucrose, fructose, xylose, arabinose, galactose and mannose content was registered by Ion Chromatography in GP culture medium at days 0, 3, 6 and 13 of the biosynthesis process. Samples were filtered through 0.2 μm syringe filters and stored at -20 °C until analysis. The determination was made with a Dionex ICS-5000 + Ion Chromatograph using the High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection technique (HPAEC-PAD). The analytic column was Dionex CarboPac PA210-4 μm, 2 × 150 mm and the protective column was Dionex CarboPac PA210 G-4 μm, 2 × 30 mm. The injection volume was of 2.5 μL and the flow rate 0.2 mL min^{-1}. The eluent gradient was isocratic, KOH 14 mM, Dionex EGC 500 KOH eluent generator was used with a Dionex CR-ATC 500 Anion Trap column continuously regenerated.

2.4. Physicochemical characterization of BC

2.4.1. Fourier transform infrared spectroscopy (FTIR)

FTIR was used to identify functional groups of BC. A Nicolet Nexus spectrophotometer provided with MKII Golden gate accessory (Specac) with diamond crystal at a nominal incidence angle of 45° and ZnSe lens was used. Spectra were recorded between 4000 and 650 cm^{-1} averaging 32 scans with a resolution of 4 cm^{-1}. The areas of absorbance bands around 710 cm^{-1} and 750 cm^{-1} were used to estimate the percentage of cellulose I_β.

2.4.2. X-ray diffraction (XRD)

XRD diffraction patterns were obtained using PHILIPS X'Pert Pro diffractometer, in θ - θ configuration secondary monochromator with CuKα ($\lambda = 0.154$ nm) and a solid state pixel detector, operating at 40 kV with a filament of 40 mA. The diffraction data were collected from 2θ values 5° to 40°, where θ is the angle of incidence of the X-ray been on the sample.

The crystallinity index (CI) of produced BC was determined by the following Eq. [30]:

$$CI\ (\%) = \frac{I_{200} - I_{am}}{I_{200}} \bullet 100 \quad (2)$$

where I_{200} is the maximum intensity of the (200) lattice diffraction at $2\theta = 22.7°$ and I_{am} is the intensity scattered by the amorphous part of the sample (the location of the amorphous material signal considered was at $2\theta = 18°$).

2.5. Superabsorbent nature characterization

2.5.1. Atomic force microscopy (AFM)

Atomic force microscopy (AFM) images of BC samples surfaces were obtained in tapping mode using a Nanoscope IIIa scanning probe microscope (Multimode™ Digital instruments) with an integrated force generated by cantilever/silicon probes, applying a resonance frequency of about 180 kHz. Cantilevers with tips of 5–10 nm in radius and 125 μm long were used. Diameter measurements of the nanofibers were made from the height images using the AFM software.

2.5.2. Water holding capacity (WHC)

Freeze-dried samples were used for WHC assays. The weighted freeze-dried samples (W_{dry}) were immersed in deionized water until constant weight (W_{wet}) [31]. The WHC was calculated with the following equation:

$$WHC\ (\%) = \frac{W_{wet} - W_{dry}}{W_{dry}} \bullet 100 \quad (3)$$

2.6. Urea release study

2.6.1. Urea loading

Freeze-dried sphere-like BCs were immersed in 1 M aqueous urea solution for 48 h. After, samples were freeze-dried again and Urea loading (UL) was measured in triplicate using the following equation:

$$Urea\ loading\ (\%) = \frac{W_L - W_{dry}}{W_{dry}} \bullet 100 \quad (4)$$

where W_L is the weight of loaded and freeze-dried BC and W_{dry} is that of the freeze-dried BC before urea loading [32].

2.6.2. Urea release profile

Two different drying methods were applied to the urea loaded BC samples, freeze-drying and oven drying at 50 °C until constant weight, with the objective of studying the effect of the drying method on the release profile. The urea release measurements were carried out placing the loaded and dried sphere-like BCs in aqueous medium at neutral pH without stirring. Afterwards, at different times between 0 and 6 h, samples were taken from the release medium and the amount of urea was measured by spectrophotometry (UV–Vis-Nir Shimadzu 3600) from the absorbance of the band at 420 nm after the reaction with 4-dimethylamino benzaldehyde and HCl as reagent [33]. The concentration of urea in the release medium was determined using a calibration curve with known urea concentrations from 0.5 mM to 50 mM. Acetonitrile was used as solvent for the reaction as published by Giraldo and Rivas [34] and each assay was carried out in triplicate.

The Cumulative urea release (CUR) was calculated from the following equation:

$$Cumulative\ urea\ release\ (\%) = \frac{W_t}{W_U} \bullet 100 \quad (5)$$

where W_t is the amount of urea released from the sphere-like BC at time t and W_U is the total amount of urea loaded into the sample.

3. Results and discussion

3.1. Characterization of culture media

3.1.1. Biosynthesis optimization and BC yield

Several GP/water ratio solutions were prepared with the purpose of stablishing the correct nutrient balance for bacterial development in GP medium. The supplementation with additional carbon or nitrogen sources of the agricultural residue based medium is a widely extended practice to increase BC production [11,31,35]. In this case, peptone and yeast extract were used as extra nitrogen and mineral sources [9,11].

Table 1 depicts the obtained results for BC production in static and agitated cultures within the studied conditions. GP concentrations above 20% (*w/v*) were also tested but growth was not observed.

As it can be observed from Table 1, GP based culture medium is suitable to produce functional BC both in static and agitated conditions. As expected, the measured glucose and fructose concentrations increased with the GP content, being similar to the sHS culture for the GP 20% (*w/v*) static culture. Attending to the results, the necessity of the nitrogen supplementation could be concluded, mainly for the lowest GP concentration media, i.e. 2.5 and 5% (*w/v*) cultures. In all the static

Table 1
Growth conditions, GP concentration, supplementation with nitrogen sources, BC production and glucose + fructose content of the different culture media tested. *Us, H* and *Us + H* refer to the application of ultrasound treatment, hot water treatment and the combination of both treatments respectively.

Growth conditions	Culture	Grape pomace % (w/v)	Nitrogen source		Glucose + fructose (g L^{-1})	BC yield (g L^{-1})
			Peptone % (w/v)	Yeast extract % (w/v)		
Static	*sHS*	–	0.5	0.5	20.0	0.75 ± 0.12
	sGP2.5	2.5	–	–	2.1 ± 0.1	–
	sGP2.5-pp	2.5	0.5	–		0.53 ± 0.02
	sGP5	5	–	–	4.4 ± 0.7	–
	sGP5-pp	5	0.5	–		0.73 ± 0.03
	sGP5-exUs + H	5	–	0.5		0.91 ± 0.06
	sGP10	10	–	–	9.2 ± 0.2	0.26 ± 0.07
	sGP10-pp	10	0.5	–		0.35 ± 0.02
	sGP20	20	–	–	20.6 ± 0.5	0.10 ± 0.04
	sGP20-pp	20	0.5	–		0.31 ± 0.00
Agitated	*aHS*	–	0.5	0.5	20.0	0.18 ± 0.02
	aGP5-ex	5	–	0.5	4.4 ± 0.7	0.58 ± 0.08
	aGP5-exUs	5	–	0.5	4.6 ± 0.1	0.60 ± 0.09
	aGP5-exH	5	–	0.5	5.5 ± 0.2	0.73 ± 0.02
	aGP5-exUs + H	5	–	0.5	5.8 ± 0.1	1.17 ± 0.21

cultures, peptone supplementation exhibited higher production yields compared to non-supplemented media [10,11]. Moreover, peptone supplementation seemed to be effective as production values were similar to the ones obtained with sHS culture medium. It is worthy to note that further increase of GP into the medium above 5% (w/v) resulted in partial inhibition of BC production, despite the higher glucose and fructose content or nitrogen supplementation [16]. Increased viscosity and concentration of polyphenolic compounds can obstruct or inhibit BC biosynthesis and cause this production decrease [36]. The disparity in glucose and fructose concentrations between the most productive GP culture medium and HS culture medium suggested that *G. medellinensis* could be metabolizing or interacting with other unidentified compounds in the GP culture medium. These results obtained in static cultures were used as reference for the design of the agitated cultures. Thus, sGP5-pp culture was selected as control for BC characterization and as starter medium for agitated cultures.

When agricultural residues are used into culture media under agitated conditions, additional factors must be taken into account for the production of BC. Indeed, the suspended solid amount was a key element for the uniform BC production in agitated cultures, and, therefore, different treatments of the GP were studied in order to reduce turbidity and increase solubility and availability of fermentable sugars. Given that acid or enzymatic treatments are expensive, require further neutralizing steps and enhance bacterial growth inhibitory compound formation [37], hot water treatment was chosen as the most suitable method for non-cellulosic monosaccharide extraction [9,11]. Besides, it has been reported that ultrasound assisted extractions of vegetal raw materials increases the efficiency of sugar recovery [38]. Ultrasounds weaken cell walls and favours mass transfer, increasing the permeability of vegetal tissues to solvents [39]. Therefore, ultrasound and/or hot water treatments were carried out to improve the nutritive profile of the GP medium.

On the other hand, peptone was discarded for the preparation of agitated cultures, since it formed aggregates in combination with the GP at those conditions and yeast extract was used as nitrogen extra source. In conclusion, four agitated GP based culture media were prepared at 5% (*w/v*) GP concentration: non-treated (aGP5-ex), ultrasound treated (aGP5-exUs), hot water treated (aGP5-exH) and that with combined treatments (aGP5-exUs+H).

As it could be observed, ultrasonic and hot water treatments favoured the sugar extraction from GP, regardless of whether they were applied separately or in combination. However, higher monosaccharide concentration was obtained for the hydrolysed media. The combination of both treatments (aGP5-exUs+H) was the most effective procedure, increasing up to 31% the content of fermentable sugar with respect to the non-treated aGP5-ex samples. Moreover, the increase of glucose and fructose concentration led to an increase of BC production. These results suggest that ultrasounds and hot water treatments are interesting alternatives to acid or enzymatic treatments when an increase of fermentable sugars for bacterial growth are desired.

Ultrasonic and hot water treatments where also applied to a static sGP5-ex culture medium in order to corroborate the positive effect observed in agitated cultures. The BC production results of the static sGP5-exUS+H culture indicated that the selected treatments were effective to improve the quality of the medium in both growing conditions. Regardless of the culture conditions and with low cost treatments, both from economic and ecological points of view, BC can be produced from small amounts of grape residue in a competitive way.

Furthermore, it is worth noting that the production of BC in agitated GP cultures was slightly higher than in static cultures. Numerous researches revealed that the production of BC was notably higher under gentle agitation conditions (100 rpm) when compared to that with static cultures [40,41]. Indeed, Revin et al. (2018) promised very high BC quality and yield values from acidic agricultural residues. It has also been reported that agitated growing conditions are the most suitable for profitable production [16]. Similarly, according to our results under agitated conditions a high amount of BC can be produced in less time than under static conditions.

It is though that the agitation process generates mutant bacteria that are not capable of synthesizing cellulose, which leads to reduce the BC production [43]. The formation of gluconic acids is another reason why cellulose production generally diminishes [44], and it has been reported that the concentration of these secondary metabolites increases in shaken cultures [40]. Nevertheless, there are *Komagateibacter* strains that perform better under agitated conditions than others as they suffer less genetic instability [41,45]. Algar et al. (2014) produced higher amounts of BC from pineapple residues in stationary cultures than in agitated cultures also with *K. medellinensis*. Hence, our yields suggest that the same bacterial strain can behave differently under static or dynamic growth conditions depending on the culture medium. This circumstance may be due to the fact that BC production is strongly influenced by the relationship between the bacterial strain and the carbon sources used [41]. This is one of the reasons why the interaction between different culture media, growth conditions and bacterial strain should be carefully studied when industrial scale BC production is desired.

3.1.2. GP culture medium sugar composition

Numerous monosaccharides and disaccharides were analysed, though only glucose, fructose and arabinose were detected with concentrations of 2.22 g L^{-1}, 2.48 g L^{-1} and 0.01 g L^{-1}, respectively. This may be caused by the filtration and dilution of the medium making difficult the detection of sugars in such small concentrations. Some previously reported studies offered higher total sugar concentrations and diversity, up to 20% (*w*/w) in the case of white grape pomace [9,46]. Nevertheless, these values refer to higher grape pomace concentrations obtained with only skins, whereas in our research seeds and stalks had also been used in the cultures. Moreover, excessive extracted fermentable sugar concentration is not required since BC can be successfully produced with small amounts of grape pomace and, as reported by Hu et al. (2013), BC biosynthesis can be progressively inhibited in agitated cultures with sugar concentrations above 20 g L^{-1}.

Fig. 1 shows the evolution of the of glucose and fructose concentration over time in static and agitated cultures. In stationary cultures, BC production usually diminishes drastically from day 13 due to the formation of inhibitory compounds [47]. On the other hand, in the case of dynamic cultures, it has been published that the BC spheres achieve their final size in nearly 60 h, so that the culture times tend to be around three to seven days [42,48,49]. For this reason, culture media was analysed by ion-exchange chromatography on days 0, 3, 6 and 13.

As it could be noted, consumption of the two monosaccharides in the case of agitated cultures is clearly faster than in static cultures. Indeed, in agitated cultures, glucose depletion occurs on the third day whereas it took six days in the case of static cultures. These results did not coincide with previously reported studies with *K. medellinensis* strain ID13488 where glucose depletion took much longer [27,31]. However, it is also true that similar consumption rates were found for *G.sucrofermentans* B-11267 using agitated cultures, from acidic agro-industrial residues [42]. Likewise, it is known that the acidic pH favours the growth of *K. medellinensis* as well as increases the consumption of glucose in *K. xylinus* agitated cultures [31,40]. In any case, this disparity in sugar consumption rate could be caused by the lower amount of initial sugar of GP and a different composition of the culture medium.

The relationship between the bacterial strain and the composition of the culture medium is closely linked to the production and properties of cellulose. In fact, within the same genus, the different cellulose-producing bacterial species show metabolic preferences for certain carbon sources and its concentrations [28,41,50]. In a comparative study between glucose, fructose and sucrose as unique carbon sources in each culture medium of *K. medellinensis*, a considerable higher production of BC was observed in glucose-based culture media [27]. So it seems that better yields are obtained in *Komagateibacter* genus when glucose is the main carbon source maybe due to the fact that it can be used both as an energy source and directly as a precursor to cellulose polymerization [27].

Similarly, as it is represented in Fig. 1, after glucose depletion, the rate of fructose consumption increased markedly in both culture conditions, indicating a variation of the main carbon source. These results reinforced the above mentioned statement that the bacterium *K. medellinensis* has priority over glucose. The additional energy requirement of the enzymatic isomerization of fructose for the subsequent polymerization to BC may explain this preference for glucose [51]. Furthermore, the stationary phase in fructose consumption could be due to the negative effect on fructokinase of using an inoculum grown in a medium where the only carbon source was glucose [52].

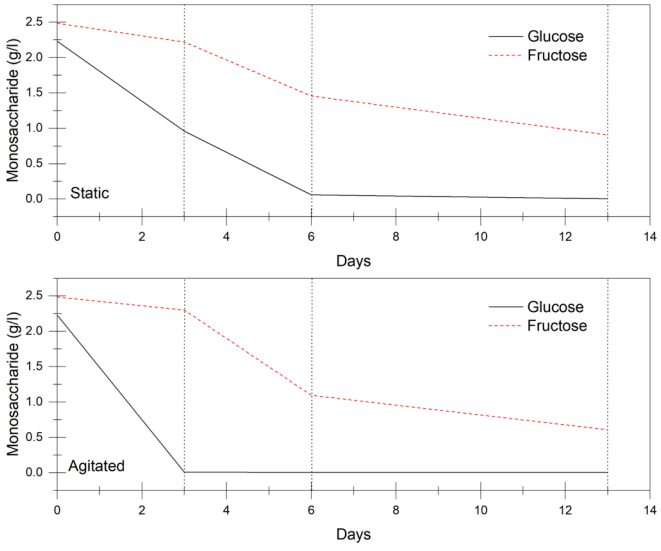

Fig. 1. Variation of glucose and fructose contents in static (a) and agitated (b) GP culture media at 5% (*w*/*v*).

3.2. Physicochemical characterization of BC

3.2.1. FTIR

The FTIR spectra of different BC samples biosynthesized under static and agitated conditions are shown in Fig. 2a. The characteristic cellulose I allomorph was identified in all analysed samples through the absorption bands at 1427, 1280 and 897 cm^{-1} [53]. The bands located at around 3300 cm^{-1} were assigned to O—H linkage stretching vibration.

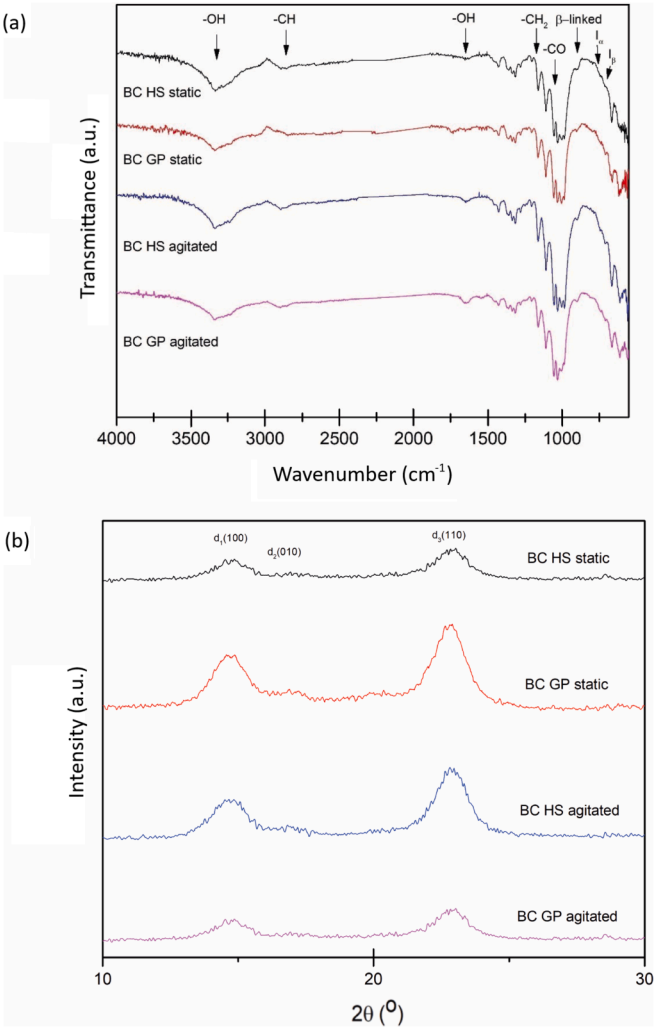

Fig. 2. FTIR spectra (a) and X-ray diffraction patterns (b) of BC in static and agitated conditions from HS and GP culture media.

The absorption bands at 2900–2880 cm^{-1} and 1460–1250 cm^{-1} corresponded to the CH and CH_2 stretching and bending vibrations, respectively. Likewise, vibrations of the C-O-C bond of the glycosidic bridges were detected by the bands at 1170–1050 cm^{-1}. The broad band at 897 cm^{-1} is characteristic of β-linked glucose based polymers. Finally, the band at around 1650 cm^{-1} was associated to the absorbed water. Regardless of the use of GP media and agitated conditions, the FTIR spectra reflect that all BC samples share the same chemical structure as well as a high purity.

As it is well known, cellulose I occurs in metastable cellulose I_α allomorph (triclinic structure) and stable cellulose I_β allomorph (monoclinic structure). The absorbance bands at 710 cm^{-1} and 750 cm^{-1}, which correspond to I_β and I_α respectively, were integrated to estimate cellulose I_β percentage (Table 2) [54]. As it was expected for BC and in contrast to vegetal cellulose, I_α is the dominant polymorph with I_β values between 37 and 41% [10]. The gentle agitation conditions do not alter the crystallization of nanofibers excessively and similar percentages of the stable I_β allomorph were obtained [50].

3.2.2. XRD

In Fig. 2b the X-ray diffraction patterns of the BC produced in HS and GP media under static and agitated conditions are shown. The patterns presented the typical crystalline structure of cellulose I with three main peaks located at $2\theta = 14.5^\circ$, 16.8° and 22.7° that correspond to (100), (010) and (110) crystallographic planes, respectively, which agrees with that observed in the FTIR spectra [55,56]. The crystallinity index (CI) calculated using the Segal equation (Eq. 2) is given in Table 2. The CI of the samples obtained from GP was slightly lower than that of the samples from HS culture medium, probably related to the lower homogeneity of the culture medium that could affect the crystallization process. CI values for BCs from agitated samples in this work were similar to those of the static samples. These findings are in agreement with the theory that under agitated conditions below 150 rpm the CI of celluloses from agitated media were similar to that of celluloses from static media [40]. This CI values were comparable to the ones reported by Algar et al. (2014) and higher than those of other studies of BC obtained from agitated media, where values between 45 and 70% were obtained [15,41,57].

3.3. Superabsorbent nature characterization

3.3.1. BC morphology

As it has been explained, agitation of the cultures causes cellulose to be biosynthetized with a different microstructure and final shape. While in static cultures gelatinous and smooth membranes formed by interconnected nanofibres are obtained, in agitated cultures cellulose is synthesized with asterisk-like, filamentous or sphere-like shape. Moreover, in agitated cultures BC presents a layered structure, porous character and high surface area [16]. The final shape of cellulose in shaken cultures depends on numerous factors such as bacterial concentration, culture time, rotation speed, bacterial strain, turbidity or pH of the medium [15]. However, the exact mechanism by which the spheres are produced remains unknown and requires further study.

In this work, special attention was drawn to both the amount of suspended solids and the rotation speed. In fact, it was found that the production of sphere-like shapes was not possible in high turbidity degrees. Thus, as described in the experimental section, GP/water mixtures were thoroughly strained and centrifuged for the culture media preparation. Regarding the rotation speed, it was found to be a key variable to obtain well defined cellulose spheres. For instance, rotation speed values of 150 rpm or higher, triggered BC in the form of 2-3 mm filaments (Fig. 3a). Reducing the agitation speed to 120 rpm or less led to the formation of large aggregates containing cellulose and impurities (Fig. 3b). Finally, at 130 rpm, sphere-like cellulose beads of 6-8 mm in size were successfully obtained (Fig. 3c). Therefore, 130 rpm was chosen as the optimal rotation speed for the synthesis of the desired sphere-like BC beads. These results are in agreement with those reported by Hu and Catchmark (2010) where sphere-like BC was obtained with diameters of 8 mm in the range of 120-150 rpm rotation speeds. The characteristic microstructure of BC obtained in agitated cultures along with the higher surface area due to the spherical shape extend the potential applications of the BC as carrier of enzymes or adsorbent of heavy metals, oils and organic solvents, among others [18].

Table 2
I_β percentage and Crystallinity Index (CI) of the different culture media in static and agitated conditions. Values are represented in triplicate with standard deviation.

Culture	Growth conditions	I_β (%)	CI (%)
sHS	Static	37.2 ± 0.3	87.3 ± 0.9
sGP5-exUs + H	Static	41.5 ± 1.0	75.2 ± 7.1
aHS	Agitated	41.1 ± 0.6	85.1 ± 2.3
aGP5-exUs + H	Agitated	41.6 ± 1.2	76.2 ± 2.8

3.3.2. AFM

Fig. 3e and Fig. 3f show the AFM images obtained for the cellulose surfaces from GP media both in agitated and static conditions, respetively. The BC height and phase images were utilized to analyse the morphology and structure of the samples. As it could be observed, in all cases, the characteristic 3D network like structure of interconnected nanofibers was observed. Regarding the culture conditions, the dimensions of the obtained nanofibers were larger in the case of static cultures compared to agitated cultures. Nanofibers obtained in static GP cultures presented a slightly larger diameter (77.8 ± 15.6 nm) than those from agitated GP cultures (63.8 ± 8.2 nm). Further, it can be noted that agitated cultures caused more irregular networks, forming more porous structures. The nanofibers synthesized from the GP culture media were slightly thicker than those published by other authors for agitated cultures from different agricultural wastes [35,50]. These morphological properties could be affecting to other features of the BC as reflected later in the WHC results.

3.3.3. Water holding capacity

As explained, BC is extracellularly synthesized into 3D networks of separated nano- and microfibrils, which enhances its surface area in comparison with vegetal cellulose. Hence, BC can retain large amounts of water without losing its structural coherence due to the numerous hydrogen bonding interactions between water and the hydroxyl groups present in the glycan chains. The hydrophilicity depends also on the interstitial spaces and porosity of the inner area in the never dried matrix [58]. The drying method also influences porosity since freeze-drying prevents pore shrinkage of the BC better than hot air drying [59].

Table 3 shows the WHC results of the BC membranes and sphere-like BCs from GP culture media after a swelling period of 48 h. Attending to the results, regardless of the culture conditions both types of BC samples showed high WHC, though higher values were obtained for the agitated medium since the surface area per mass unit increased. Indeed, WHC values were 60% higher when BCs from GP medium were biosynthesized under agitation. As it can be observed in the AFM images (Fig. 3) and attending to the CI values (Table 2), the 3D network formed in agitated media presented a more porous of less crystalline cellulosic structure. It has been previously reported that agitated conditions could affect BC microstructure decreasing the CI, degree of polymerization and crystallite size, among others [45]. Therefore, apart from the increase of the surface area as a result of the spherical shape, these changes in BC microstructure and porosity could be conferring higher WHC capacity to the system. In the same way, as the fiber diameter decreases slightly with agitation, the results indicate that finer and longer nanofibers positively influenced the WHC [31,60]. As a consequence, all GP samples showed higher WHC values compared to those reported by other authors, thus confirming their superabsorbent nature [35,58,61,62].

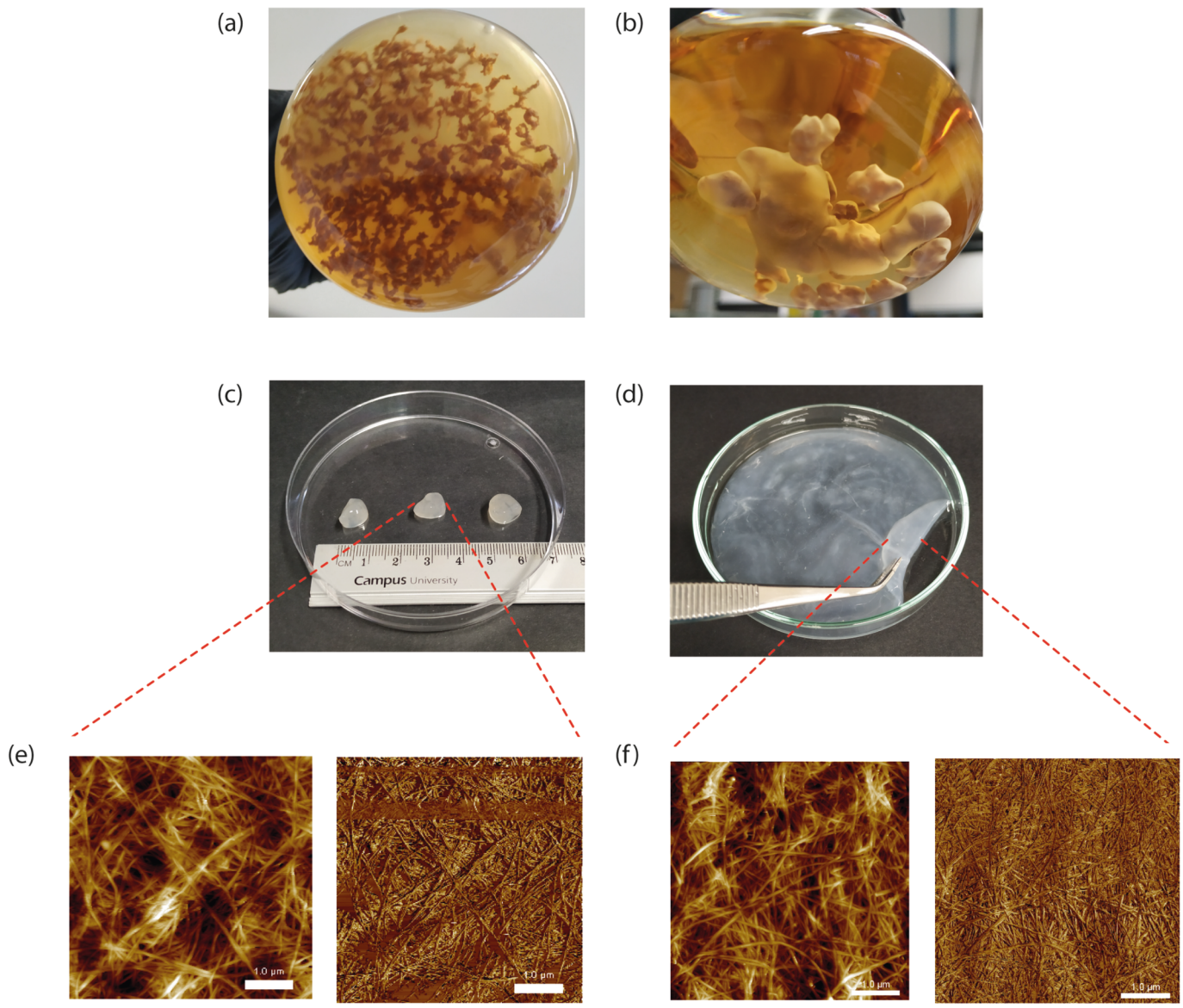

Fig. 3. BC samples produced in agitated GP cultures at rotation speeds of (a) 150 rpm, (b) 120 rpm and (c) 130 rpm. The image (d) corresponds to a BC membrane from static growing conditions and GP culture medium. Height and phase AFM images of BC samples from agitated GP and static GP culture media are represented in (e) and (f), respetively.

Table 3
Nanofiber diameter and Water holding capacity (WHC) of BC from GP culture media in static and agitated conditions.

Culture	Growth conditions	Nanofiber diameter (nm)	WHC (%)
sGP5-exUs + H	Static	77.6 ± 13.7	4870 ± 350
aGP5-exUs + H	Agitated	63.8 ± 8.2	7860 ± 35

The added superabsorbent capacity of the sphere-like BCs expands the applications of these beads and it enhances the usefulness of agitated growing conditions. The high WHC value of BC is an interesting feature that has already been successfully exploited mainly for the food and biomedical sectors [63,64]. Thereby, the versatility and high WHC contribute to a more effective grape pomace valorisation. Given the properties of the sphere-like BC, its applicability as soil water optimization agent in agriculture is proposed, considering the lack of knowledge about the potential of BC in this field.

3.4. Urea release study

The ability of BC synthesized from GP to absorb and release a fertilizer was evaluated. Urea was chosen as organic fertilizer since it is the most widely used nitrogen containing fertilizer and it was loaded into the freeze-dried sphere-like BCs from GP cultures by immersion. The urea loading capacity of the sphere-like BC from GP culture was calculated to be higher than 375%. This high loading capacity would be related both to the spherical shape and high porosity of the samples, that resulted in great WHC as explained before. Moreover, the loading efficiency was enhanced by the hydrophilicity of urea and the hydrogen bonding interactions between its $–NH_2$ groups and the –OH groups of cellulose.

After the freeze-drying and oven drying processes, the absorbance of samples taken from the release medium at different time intervals was analysed. Irrespective of the drying method, the release of urea occurred immediately and reached 100% in 30 min. Consequently, BC is capable of absorbing large amounts of urea, retaining and releasing it when surrounded by aqueous environments.

4. Conclusions

In this work, sphere-like BCs were successfully biosynthesized from GP based culture media contributing to the revaluation of this agro-industrial residue. The growth of *K. medellinensis* was analysed and optimized under both static and agitated culture conditions. In this sense, mild agitating conditions of 130 rpm were found to be effective in sphere-like BC production. Furthermore, the use of ultrasound and hot water pretreatments in the GP improved the availability of nutrients in the culture, and consequently, larger BC production yields were obtained. As a result, sphere-like BCs of great purity and high crystallinity were obtained, as it was proved in their physicochemical characterization. To assess the applicability of BC spheres in agriculture, their superabsorbent nature and their ability to retain and release a fertilizer were favorably evidenced. Thus, they could be considered as dual systems, able to help in soil water retention and release urea in the presence of water until their degradation. Finally, future research lines will elucidate the biodegradability rate of sphere-like BCs as well as their performance in the agricultural field.

Ethical approval, ethical standards and conflict of interest

Hereby, I, Nagore Gabilondo, consciously assure that for the manuscript Superabsorbent bacterial cellulose spheres biosynthesized from winery by-products as natural carriers for fertilizers the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

7) There is no conflict of interest.

8) There are not animal studies or human participants in the study.

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