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Review

Progress in bacterial cellulose matrices for biotechnological applications

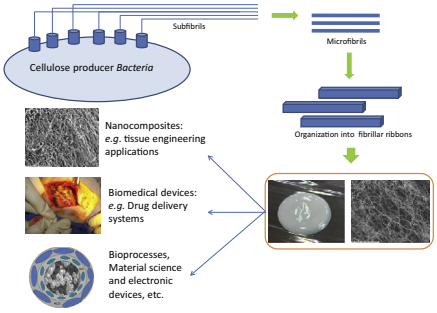
Maximiliano L. Cacicedo [a], M. Cristina Castro [b], Ioannis Servetas [c], Loulouda Bosnea [c], Konstantina Boura [c], Panagiota Tsafrakidou [c], Agapi Dima [c], Antonia Terpou [c], Athanasios Koutinas [c], Guillermo R. Castro [a,*]

[a] Nanobiomaterials Laboratory, Applied Biotechnology Institute (CINDEFI, UNLP-CONICET CCT La Plata), Department of Chemistry, School of Sciences, Universidad Nacional de La Plata, CP 1900 AJL Ciudad de La Plata, Provincia de Buenos Aires, Argentina
[b] School of Engineering, Universidad Pontificia Bolivariana, Circular 1 # 70-01, Medellín, Colombia
[c] Food Biotechnology Group, Department of Chemistry, University of Patras, 26500 Patras, Greece

HIGHLIGHTS

- Cellulose producing microorganisms: the *Komagataeibacter* genus and main characteristics.
- Cultivation and media to produce bacterial cellulose.
- Modification of bacterial cellulose: *in-situ* and ex-*situ* techniques.
- Bacterial cellulose in biotechnological processes: enzyme and cell immobilization.
- Multilayer bioreactors based on bacterial cellulose matrices.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacterial cellulose (BC) is an extracellular polymer produced by many microorganisms. The *Komagataeibacter* genus is the best producer using semi-synthetic media and agricultural wastes. The main advantages of BC are the nanoporous structure, high water content and free hydroxyl groups. Modification of BC can be made by two strategies: *in-situ*, during the BC production, and *ex-situ* after BC purification. In bioprocesses, multilayer BC nanocomposites can contain biocatalysts designed to be suitable for outside to inside cell activities. These nanocomposites biocatalysts can (i) increase productivity in bioreactors and bioprocessing, (ii) provide cell activities does not possess without DNA cloning and (iii) provide novel nano-carriers for cell inside activity and bioprocessing. In nanomedicine, BC matrices containing therapeutic molecules can be used for pathologies like skin burns, and implantable therapeutic devices. In nanoelectronics, semiconductors BC-based using salts and synthetic polymers brings novel films showing excellent optical and photochemical properties.

1. Introduction

Bacterial cellulose (BC) was first discovered two centuries ago, but only in the last decades with the development Green Chemistry and nanotechnologies is gaining high attention by research community in both academic and industrial fields. BC is β-glucan biopolymer composed only of β-1,4-glucopyranosyl units with polymerization degree up to several millions. BC is made of subfibrils of 1.5 nm assembled into nanofibrils of 2–4 nm (but up 25 nm) width composed of 10–250 single polymeric chains and 1–9 nm length equivalent from 2000 up 18,000–20,000 glucose units organized into nanoribbons of 40–60 nm width (Fig. 1) (Ruka et al., 2014). The nanoporous network of BC plus the simple techniques

* Corresponding author.
E-mail address: grcastro@gmail.com (G.R. Castro).

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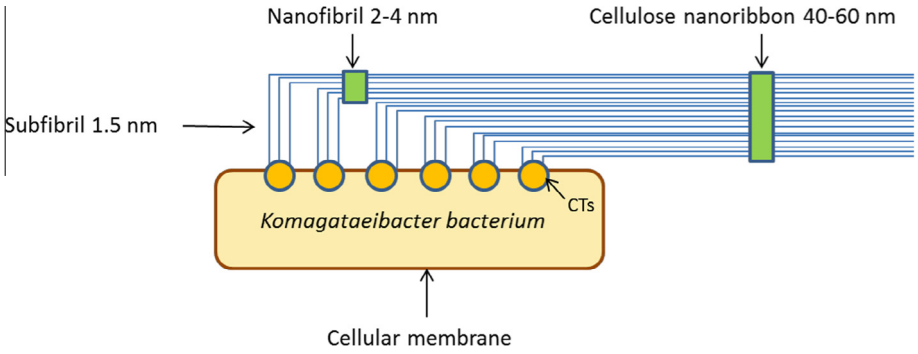

Fig. 1. Schematic image of BC nanoribbon configuration (modified from Chawla et al., 2009).

for purification and modification makes bacterial cellulose very attractive material for many biotechnological purposes. BC is very versatile and can be obtained in the forms of nanofibrils, micro- and nano-particles (by chemical or enzymatic modification), and as biofilm matrix with different degrees of crystallinity just by choosing appropriate microbial strain and/or combined within different fermentation strategies. Chemical modifications of BC by classical redox reactions are also available because of simple and unique micro- and nano-fibril structures. Besides, the novel trends in Chemistry using soft and environmental benign techniques are making green methods for BC modification more attractive. Two main green methods were developed: *in-situ* BC modification that implies the addition of exogenous material(s) to the medium culture at starting time of bacterial growth, *e.g.* polymers, detergents, etc., during the fermentation process. The novel BC structure is the result of the nanofibrils entanglement with the exogenous molecules making matrices with new features and biophysical properties. Alternatively, *ex-situ* modification is based in the modification of BC matrix after production and purification followed by the addition of exogenous molecules (*e.g.* salts, polymers, etc.) over the BC surface creating a novel layer with different physicochemical and biological properties (Shah et al., 2013).

Typical examples of the diverse cellulose applications are nanofiltration devices, light amplifier system for single gene detection, sunscreen in cosmetics and composites made within synthetic polymers using modified BC. The main advantages of BC are the high mechanical resistance, high water content and nanodimentional network formed when the nanofibrils of about 80 nm are entangled. Furthermore, tubular cellulose (TC) can be obtained after delignification of cellulosic materials for many purposes (Koutinas et al., 2012). Recently, it was proposed the use of BC for the development of two layer fermentation through its composite of Tubular Cellulose/Starch gel (TC/S), having different microorganisms in each layer to avoid biological competition amongst them (Servetas et al., 2013). However, tubular cellulose contains mainly micro-tubes and low concentration of nanotubes (Gialleli et al., 2014). Likewise, it is well-known that bioprocessing is done through cell inside activities by transferring nanowires to cell specific locations, which liberates bioactive compounds through microfluidic or photoactivable technics (Hilmer and Strano, 2010).

The aims of the present review are to show the state of the art in the production of and the recent technological advances of bacterial cellulose in the area of biotechnology. Also, to propose the use of bacterial cellulose as support of multi-layer nanocomposite production to encapsulate different bioactive materials, in order to be used (i) in bio-processing through cell inside and separately outside activities and (ii) as bioreactor bio-processing, and in nanomedicine and electronics.

2. Properties of bacterial cellulose

Plant cellulose is produced as lignocellulosic polymer which means that cellulose molecules are strongly associated with others, like lignin, hemicellulose and other substances. In addition, the cellulose content in plants depends on the natural sources. As an example, in cotton the amount of cellulose is around 90% meanwhile in wood the content is reduced to about 50%. All these accessory molecules that go along with cellulose have specific functionalities in plant physiology. However, they are considerable amount of impurities when plant cellulose required to be used for certain applications in where fine molecular tuning is required and/or sensitive area such as in biomedicine. Also, purification and isolation of plant high purity cellulose is a difficult task involving complex procedures such as mechanical treatment followed by pre-treatments based on the use of harsh chemical combined or not with enzymatic processes. All the cellulose purification processes at industrial scale are high energy consumers, approximately 1000 kWh/Ton, unfortunately are still high and expensive. Moreover, environmental issues required to be attended due to the toxicity of byproducts during the purification process (Siró and Plackett, 2010). On the other side, BC is produced in a highly pure form and the purification process is simpler, cheaper and environmentally friendly (Shi et al., 2014). Most of the laboratories who work with bacterial cellulose utilize very easy purification processes, usually with alkalis (Cacicedo et al., 2015).

BC is a biomaterial only composed of glucose units and water, its mechanical behavior can be compared with other complex and synthetically produced polymers or fibers. BC tensile strength can be in the range of 200–300 MPa, and its Young's modulus up to 15–35 GPa. For example, polypropylene (PP) has a lower tensile strength of 30–45 MPa and a Young's modulus of 1,0–1,5 GPa compared to BC (Ruka et al., 2014). These mechanical properties are a direct consequence of the crystalline nano- and micro-fibril BC structures. Furthermore, the combination of high crystallinity and high water content are also responsible of BC thermal stability (Qiu and Netravali, 2014). This is a key feature that makes possible the BC sterilization by an easy heating process, *e.g.* autoclaving. A simple and cheap way of biomaterial sterilization is advantageous in the biomedical field since not too many polymers can be heated above 100 °C without changing their biophysical properties.

3. Cellulose producing microorganisms

Bacterial cellulose was first identified by Brown in 1886 during vinegar fermentation. Brown noted that the substance named as "vinegar plant" or "mother" had the same structure, composition and reactivity than plant cellulose. The most common cellulose producing bacteria are members of the family *Acetobactereaceae*

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and particularly belongs to the genera *Komagataeibacter* (former *Acetobacter* first and later as *Gluconacetobacter* genus), *Agrobacterium*, *Rhizobium* and *Sarcina* recently revisited (Ruka et al., 2014).

Besides, the major cellulose producers are microorganisms belonging to the *Komagataeibacter* genus, recently created by reclassifying the *Gluconacetobacter* genus on the basis of 16S rRNA sequence phylogeny, phenotypic, ecologic and chemotaxonomic characteristics. The *Gluconacetobacter* genus was subdivided into two and one is the genus *Komagataeibacter* generated by the *Gluconacetobacter xylinus* group, with the most studied and type *Komagataeibacter xylinus* species. The genus *Komagataeibacter* is physiologically characterized by the production of acetic acid from ethanol, the oxidation of acetate and lactate to carbon dioxide and water, able to growth in presence of 0.35% (w/v) acetic acid, without production of 2,5-diketo-D-gluconate from glucose and morphologically without motility (Yamada, 2014).

The *Komagataeibacter* species is defined as Gram-negative, strictly aerobic, living mainly in fruits and vegetables in decomposition process, they are able to convert common carbon sources such as glucose, glycerol, sucrose, fructose, mannitol and others at temperatures between 25 °C and 30 °C at 3–7 pH range but showing different properties and yields.

The bacterial cellulose synthesized by *Komagataeibacter* spp. is identical to that produced by plants in their molecular and polymeric structure, but with high crystalline structure and also chemically pure (free of lignin and hemicellulose) (Ruka et al., 2014). The bacteria produces cellulose at the interface of air and media culture as a film for a flotation mechanism, allowing the bacteria remain in the air/liquid close to the interface to get the necessary oxygen for their metabolism. On the other hand, the film makes a physical barrier that protects against UV radiation, increases the ability to colonize other substrates and keeps its hygroscopic nature, allows them to retain moisture and prevent dehydration (Cacicedo et al., 2015).

The synthesis of the bacterial cellulose is based on a synchronic, accurate and regulated process steps, involving a large number of enzymes and protein complexes. The process includes the transport of the carbon source, *e.g.* glucose, from the outside into the cell, the synthesis of uridine diphosphoglucose (UDPG) via glucose 6-phosphate and glucose 1-phosphate, followed by polymerization of glucose in chains by cellulose CS synthase. Then nascent cellulose chains are extruded through the cell membrane. A single cell of *Komagataeibacter* spp has between 50 and 80 pores or complex terminals (CTs) of 3.5 nm in diameter for extruding cellulose from their cell membrane. Through the pores, the bacteria extruded polymer chains that join to form subfibrils (10–15 channels). The nascent cellulose chains associate with each other forming subfibrils with a width of 1.5 nm (Vitta and Thiruvengadam, 2012). The subfibrils are also autoassembled into nanofibrils (3–4 nm thick) which in turn are joined to form a ribbon with 40–60 nm width and a 3–8 nm thickness (Fig. 1) (Shi et al., 2014). The fibrils are exhibiting an extraordinary high surface area and building an exceptional 3D network structure. Due to this supramolecular structure, cellulose can be considered as semicrystalline polymer that in its original or "native" state presents polymorphism I with a mixture of two allomorphisms Iα and Iβ and Iα/Iβ ratio which varies according to the biological source and culture methodology. For example, vegetable cellulose is rich in allomorphism Iβ, meanwhile BC is rich in allomorphism Iα. The spatial arrangement of the pre-microfibril aggregation provides a high crystallinity for BC up to 80–90%, while for plant cellulose the values goes between 40% and 60% (Huang et al., 2014). Organization of BC nanofibrils leads to stronger hydrogen bonding in comparison with plant cellulose. Added to that, the bacterial cellulose chains have a very high degree of polymerization (can be up to 20,000 glucose units) (Chen et al., 2013). This network structure is plenty full of hydrogen bonds that can easily interact with other molecules such as polymers, nanoparticles or even water. Indeed, BC has a very high capacity of water content (more than 99%) specifically thanks to the interaction by hydrogen bonding. The BC crystallinity, a function of hydrogen bonding, is one of the most important causes of the mentioned mechanical properties (Ruka et al., 2014).

At the time of cell division, a branch on the ribbon is generated producing a dense network which finally forms the macroscopic membrane observed on the surface of the liquid culture media. The branching network is affected by proliferation rate of *Komagataeibacter* cells, which also depends on environmental factors such as: oxygen tension, carbon source, media, physicochemical setup and modifying agents among others (Cacicedo et al., 2016).

BC production have attracted high interest because of their properties, which makes it an attractive material for applications in several fields from bioprocesses to cosmetic, medical, paper, packaging, electronics, etc. The main challenge is still to introduce the BC into the market with a competitive price and production. This is influenced mainly by the low efficiency and high costs of raw materials rather than for the production of the BC (Castro et al., 2012).

4. Bacterial cellulose production

Bacterial cellulose production has traditionally been performed using defined culture medium, commonly named HS honoring Hestrin and Shramm who develop the media in 1954. The HS medium is composed of glucose, peptone, yeast extract, disodium phosphate, citric acid and adjusted to pH 6. However, changes in carbon source, nitrogen source, pH and inductors starting from this culture medium allowed to modify the BC productivity (Castro et al., 2015). For example, *Komagataeibacter xylinum* (the most referenced and used strain worldwide) was grown in liquid medium using many carbon sources such as amylose and amylopectin, fructose, glucose, glycerol, lactose, malic acid, maltose, mannitol, mannose, methanol, rhamnose, ribose, sorbose, sucrose among others and recently reviewed (Ruka et al., 2014). However, *Acetobacter* sp. V6 cultivated in 3.0% glycerol as carbon source in a synthetic medium showed 3.8 yields higher than the same medium supplemented with glucose (Jung et al., 2010). Considering that glycerol is the main waste in the biodiesel production, the reduction of costs by the carbon source could be very attractive form economic point of view. Also, the authors reported an increase of 9% in the crystalline structure of BC biofilm by the presence of glycerol in the culture and compared with glucose as carbon source (Jung et al., 2010).

Enterobacter amnigenus GH-1 isolated from rotten apple and cultivated in HS medium supplemented with different monosaccharides and disaccharides displayed BC yields differences lower than 10%, with the exception of glycerol (about 50% yield lower). Meanwhile, when the HS medium was supplemented with orange and pineapple juice and molasses in place of glucose showed approximately 48%, 31%, and 17% higher yields as compared to the glucose under the same experimental conditions. The results are suggesting the specific requirement of other factors rather than carbon source as inductors for BC production by *E. amnigenus* GH-1 (Hungund and Gupta, 2010).

Sucrose, a disaccharide composed of α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside, as microbial carbon source produce low BC yields during the first four days of *K. xylinum* ATCC 53524 culture compared to glucose, but the BC is higher after the fifth day of culture in a rich media buffered at pH 5 (Mikkelsen et al., 2009). The low efficiency of BC yields at the beginning of the *K. xylinum* culture supplemented with sucrose was attributed to the absence of sucrose synthase (E.C. 2.4.1.13), enzyme responsible

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for the sucrose conversion into UDP-glucose, a common precursor of BC (Nakai et al., 1999). Similarly, fructose or glucose combined or not with sucrose as carbon source are showing high and low BC yields respectively. Particularly, fructose is giving high yields of BC because of the final products are cellulose and CO_2, meanwhile low yield of BC was observed using glucose because of the carbon skeleton is redirected mainly to the gluconate synthesis by glucose dehydrogenase lowering the pH (Ruka et al., 2014).

Regarding to the nitrogen sources, the highest BC productivity was reported using yeast extract compared to others such as peptone, polypeptone, tryptone, corn steep liquor and ammonium sulfate (Ruka et al., 2014).

Recently, many researchers have also start using agroindustrial wastes to develop a culture media showing an increase of cellulose production with lower costs. The high BC yield was attributed to the amount of additional micro and macro nutrients in the waste are summarized in Table 1.

Another cultivation strategy was based on the addition of inducers to activate the energy metabolism in the microorganism and/or reduce the formation of metabolic by-products, which affects the BC production. Table 2 shows some of inductors and their effects in the BC production. For BC production, different fermentation strategies have been studied such as batch, fed-batch, continuous, under agitated o static conditions. However, the physical characteristics, properties and morphology of cellulose will be different in each case. The fermentation method used depends on the strain and type of cellulose required, since the supramolecular structure could be altered by the production method (Table 3).

Table 1
Different substrates used for producing BC.

Substrates	BC yield	References
Beet molasses	Increased by 31% compared to glucose media used	Keshk and Sameshima (2005)
Coconut water and pineapple juice	Comparable efficiency with HS medium	Almeida et al. (2008)
Corn cob acid hydrolysate	4 g/L	Huang et al. (2014)
Crude glycerol and sunflower meal hydrolysates combination	13.3 g/L	Tsouko et al. (2015)
Date syrup	3 times higher than sucrose	Moosavi-Nasab and Yousefi (2011)
Date syrup	1.2 g/L	Mohammadkazemi et al. (2015)a
Flour-rich hydrolysates (from confectionary industries waste)	13 g/L	Tsouko et al. (2015)
Konjac flour	3 times higher than glucose	Hong and Qiu (2008)
Lignocellulose biorefinery sewage	20 g/l	Cavka et al. (2013)
Maple syrup	Comparable efficiency (maple syrup 1.51 g/l fructose 1.60 g/l)	Zenga et al. (2011)
Melón peel	Highest amount achieved 8.34 g	Mohamed (2010)
Molasses	1.6 g/L	Çakar et al. (2014)
Orange or pineapple juice	Increased 48% or 31%	Hungund and Gupta (2010)
Pineapple juice and peel	Comparable efficiency with HS medium	Castro et al. (2012)
Sugar cane molasses	Increased by 190–255% Increased 106%	Premjet et al. (2007) Hungund and Gupta (2010)
Wastewater rice wine distillery	The highest production was 6.31 g/l	Wu and Liu (2013)
Wheat straw hydrolysate	8.3 g/L	Chen et al. (2013)

5. Bacterial cellulose modifications

Several methods for bacterial cellulose modification have been studied and described in the last years. The BC modification methods can be grouped based on the type of the technique. In this sense, the *in situ* method utilizes the addition of exogenous molecules to the BC media at the beginning of the microbial cultivation (Cacicedo et al., 2016). On the other hand, the *ex-situ* method consists in the addition of the extra materials once the BC has been synthesized and purified (Shah et al., 2013; Cacicedo et al., 2015).

5.1. In situ modification method

Many exogenous molecules have been added to the culture media during the production of BC interfering with the nanofibrils crosslinking in order to provide materials with novel properties. The main objective is to introduce new properties to the matrix by modifying the intrinsic biophysical properties of the bacterial cellulose. These "accessory" molecules become part of the BC nano- or micro-fibril network by interacting usually with many –OH moieties of glucose, present in BC chains, building new interconnected hydrogen bridges. A wide range of materials from different nature can be used for these purposes. Usually hydrophilic and water soluble molecules are the chosen, but some reports with hydrophobic material have been published as well. For example, BC films were produced in the presence of different molecules like Tween 80, hydroxypropylmethyl cellulose (HPMC) and carboxymethyl cellulose (CMC) among others. One of the main objectives of these modifications was to obtain a biopolymeric film with improved rehydration ability, which is one of the limitations of BC membranes after drying. The authors described the intermolecular interactions between BC fibers and the exogenous molecules by combining the gelation mechanism of the two different molecular components. In this way, polymers of high molecular weight like CMC and HPMC cannot enter to the crystalline zone between the subfibrils. However, they can modify the assembling of the microfibrils by steric hindrance avoiding the formation of H-bridges among them. Therefore, new hybrid BC matrices with HPMC or CMC showed a weakened and reduced crystalline structure contributing to the formation of greater amorphous regions that facilitate the absorption of water during the rehydration process (Huang et al., 2010). The new BC hybrid materials result in the formation of interpenetrating networks corresponding to the Morris model I that involves a network formed by active polymers which are structurally cooperative due to the entwining of the two networks (Morris, 1985). It is extremely interesting how the synthesis of BC composites by *in situ* method can be understood by studying the intermolecular relationships between BC chains and the exogenous molecules. The description of the interaction between two polymers forming a novel matrix was defined as Interpenetrated Polymer Network (IPN), in where a polymeric network is produced by the synthesis and crosslinking of BC with other polymer (Cacicedo et al., 2016).

Recently, glyoxalization of BC was performed by the *in-situ* method. Very low amounts of glyoxal precursors (a non-formaldehyde crosslinking agent) were added to *Gluconacetobacter medellensis* culture with the aim to synthesize a highly crosslinked BC. The crosslinking was induced by thermal curing after synthesis, and a stretching in the ribbons of the network and their fusion could be observed. A curious point is that the authors no noticed any change in the BC crystallinity which are indicating that the modification was only made in the surface of the biomaterial (Castro et al., 2015).

Also, *in-situ* modification of BC with water insoluble polyhydroxyalkanoate was performed with the aim to making a fully

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Table 2
Different inducers used for BC producing.

Inducer	Concentration	Microorganism	Medium	Culture type	Comments	References
Acetic acid	20 g/l	*Acetobacter xylinum*	GPY	Batch	BC production increases 4 times	Toda et al. (1997)
Agar	0.2–1.0% (w/v)	*Acetobacter xylinum BPR 2001*	CSL-Fru medium	Stirred batch	BC production improves by agar adding due to increased viscosity and free cells	Bae et al. (2004)
Ascorbic acid (vitamin C)	0.50% (w/w)	*Gluconacetobacter xylinus*	HS medium	Batch without stirring	The presence of Vitamin C in the HS medium increases 1.52 BC production and reduce gluconic acid production which diminish cell viability	Keshk (2014)
Avicel	0.8% (w/v)	*Gluconacetobacter xylinus*	Modified CSL-Fructose medium	PCS-RDB	Improved production in 116% compared with the control	Lin et al. (2016)
Ca^{2+}	–	*Acetobactoer aceti* subsp. *xylinus*	Konjac hydrolyzate powder	Batch	BC production increased 1.5 times by addition of Ca^{2+}	Hong and Qiu (2008)
$CaCO_3$	0.5% (w/v)	*Rhodococcus sp. MI 2*	Modified HS	Static	BC production increased twice from 3.7 g/L to 7.4 g/L	Tanskul et al. (2013)
CMC	0.8% (w/v)	*Gluconacetobacter xylinus*	Modified CSL-Fructose medium	PCS-RDB	Improved production in 80% compared with the control	Lin et al. (2016)
Coffee waste	1:1 (w/v) dilution	*Gluconacetobacter hansenii*	Coffee cherry husk	Stirred batch	BC production increased 3 times than that found for HS. This may be due to the polyphenols contained in the residue, which act as stimulators of BC production, preventing the c-di-GMP degradation by the enzyme phosphodiesterase	Rani and Appaiah (2011)
Ethanol and acetic acid	1.5% ethanol, 1.0% acetic acid				BC production increased 1.7 times compared without these supplements	
Ethanol	4–10 g/l	*Acetobacter xylinum subsp. sucrofermentans*	CSL-Fru medium	Continuous	10 g/l Ethanol enhanced 50% BC production.	Naritomi et al. (1998a)
Ethanol	2.00%	*Gluconacetobacter hansenii*	BM	Batch	Ethanol functions as an energy source for ATP generation in the early stages of fermentation, resulting in decreased production of glycerol, a major by-product which is lowering the BC yield	Li et al., 2012
Glucuronic acid oligomers	1% (w/v)	*Gluconacetobacter hansenii PJK*	MAE	Shaked flask	10.45 g/L compared with 7.4 g/L obtained in control medium	Ul-Islam et al., 2013
Lactic acid	4.5–12.5 g/l	*Acetobacter xylinum subsp. sucrofermentans*	CSL-Fru medium	Continuous	Lactate increases BC production. But the BC production decreased at 20 g/l of higher lactate. Lactate stimulates cell growth during early stages of growth	Naritomi et al. (1998b)
Lactic acid	0.15% (v/v)	*Acetobacter xylinum subsp. sucrofermentans*	4% w/v Fructose % w/v KH_2PO_4 0.025% w/v $MgSO_47H_2O$ 1% v/v sln. salts 1% v/v sln. vitamins 0.24% nitrogen source	Stirred batch	Lactic acid stimulates cell growth and BC production is increased 4–5 times	Matsouka et al. (1996)
Amino acids Methionine	Amino acids 0.005% (w/v)				Amino acids increase 10 times BC production. Methionine is essential to achieve high BC production	
Microparticles (diatomaceous earth, silica gel, etc.)	Optimized	*Acetobacter sp.*	–	Stirred batch	Microparticles in the media tripled the BC production	Vandamme et al., 1998
Sodium alginate	0.4 g/L	*Gluconacetobacter xylinus*	Modified CSL medium	Shaked flask	BC production of 8.25 g/L compared with 1.7 g/L obtained in control medium	Atwa et al., 2015
Sodium citrate	2.00%	*Gluconacetobacter hansenii*	BM	Batch	The citrate supplementation results in the reduction of byproducts (e.g. acetic acid, pyruvic acid) increasing BC production	Li et al., 2012
Xanthine	0.12% infusion	*Acetobacter xylinum*	HS medium	Batch	BC production increased with *Camellia sinensis* (35.3 g) and *Paulina cupana* (32.7 g). For *Coffea arabica* (14.0 g) was obtained; *Theobroma cacao* (11.6 g); *Kola sharp* (8.6 g) and *Ilex paraguayensis* (7.5 g)	Fontana et al., 1991

degradable nanocomposite system (Ruka et al., 2013). Poly-3-hydroxybutyrate (PHB) was supplemented to the *K. xylinus* batch culture. Addition of exogenous polymers (*i.e.*, PHB or alginate) not only affected BC production, but also changed the morphology and crystallinity of BC as observed in a SEM picture (Cacicedo et al., 2016).

Another interesting approach is using *in-situ* modification of BC with carboxymethylcellulose (CMC) producing a tube-formed matrix with the objective of using it as a biofilter for blood proteins (Orelma et al., 2014). After BC/CMC production and purification, the matrix was modified with the conjugation of anti-human serum albumin (*anti-HSA*) affibodies to the carboxyl groups via

Table 3
Main characteristics of BC fermentation process.

Type of cultivation	Form of BNC	Type of bioreactor	References
Stirred	Airlift reactor formed a unique ellipse pellet (BC pellet) BC was doubled	Batch, 50-L internal-loop airlift reactor.	Chao et al. (2000)
Stirred	BC production was increased. The method is very useful for the mass production	Batch, airlift-type bubble column bioreactor	Song et al. (2009)
Stirred	The impellers such as Maxblend and gate with turbine were suitable for BC fermentation, the production rate and yield of BC were dependent on KS and the oxygen consumption rate	Vessel with different agitation impellers and aeration	Kouda et al. (1997)
Static	The shape of the membrane was flat sheet, flat sack, tube and cylindrical balloon. Production rate of cellulose as well as its yield on consumed glucose by the bacteria grown on the flat type membranes was approximately ten-fold greater than those on the non-flat ones in spite of the same membrane thickness	Membrane bioreactors of oxygen permeable material	Bäckdahl et al. (2011)
Static	This system produced 2 mm/day of BC, which is approximately 9 g/day of dry BC	Fed-batch reactor with an aerosol spray system	Hornung et al. (2007)
Static	Planar BC fleeces and foils with a freely selectable lengthand an adjustable height. Yield of 0.5–1.5 mm/day	"HoLiR" (Horizontal lift reactor), semi-continuous cultivation	Kralisch et al. (2010)

covalent crosslinking with the traditional EDC/NHS method (*i.e.* 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide). Affibodies, are promising small engineered proteins with high affinity and binding specificity for defined substrates and high potential in many fields, particularly in diagnostic and therapeutic (Löfblom et al., 2010). The presence of CMC in the BC network reduces irreversible structural changes during the drying process. Similar results were previously reported (Huang et al., 2014). Finally, the specific protein detection was verified with fluorescence-stained human serum albumin (HSA) demonstrating the potential of the hybrid BC system and open up new ways in the field of biofiltration (Orelma et al., 2014).

5.2. Ex situ modification method

After BC production and purification, structural modifications can be achieved by the *ex-situ* methodology. The most often used protocol is the simple immersion of pure BC matrix into a solution where it can interact with exogenous molecules, named also as accessory material. One of the most relevant points about this method is that the original BC keeps its native structure almost unchanged. In addition, only nanosize materials can be impregnated into the BC matrix just by diffusional mechanisms to get through the network pores. The main drawback of the *ex-situ* method is reversibility of the procedure which depends not only of the interaction of the nano-object with the BC matrix but also on the physicochemical environmental conditions (Shah et al., 2013).

6. Uses of bacterial cellulose

6.1. Bioprocess – biotransformations

Bacterial cellulose can play a dual role to be an "enzymatic matrix" and also as support for other polymers containing other molecules, cellular structures and also cells. In this sense, BC can be used in biotransformations as multi-enzymatic fixed bed reactor. For example, after one enzyme immobilization on BC matrix, a second layer of other polymer likes viscous starch (VS) containing a second enzyme can be deposited over the BC surface. The procedure is very simple and involves the dissolution of starch in hot water, following by cooling and addition of other enzyme. Subsequently, the slurry is filtered or centrifuged and the solid mass is dried in a thin layer. The dry bio-composite two layer enzymatic biocatalyst of BC/VS is added and agitated in a viscous solution of alginates (ALG) containing a third enzyme type. It is prepared similarly as aforementioned described technique for VS. Then it is filtered or centrifuged and after drying can be used as a three layer composite at least three different biocatalyst of BC/VS/ALG matrix.

The two or three layer composite containing different biocatalysts is proposed in order to produce in the same bioreactor two or three products or it required a sequential reactions for the synthesis of an specific molecule. The main advantages of the multiple layer BC matrixes are the increase of productivity, decrease size of the factory and investment required for installation, operational costs and time. In order to be used this composite biocatalyst for food and pharmaceutical applications, it is required the biopolymeric composite to be friendly to human health, e.g. GRAS (Generally Regarded As Safe by FDA, USA). For that reason, the choice of biopolymers such as BC, VS and ALG fulfilled the regulatory requirements.

Similarly, the designed nano-composite of one layer and/or two layers supported by the BC, can be used for inside cell activities by penetrating cell membranes with nanofibrils. BC mutlilayer-matrixes can be partially hydrolyzed by encapsulating enzymes like cellulases, amylases and/or alginate lyases (Islan et al., 2015). The immobilization of cells can be done on the surface of the single layer of BC, while enzymes can be immobilized inside the BC network. The three polysaccharides BC, VS and ALG can make three different layers with high stability. However, one of the main drawbacks of this technique is associated with diffusional barriers of substrates and products crossing individual layers which are directly related not only to the number of layers in the BC matrix but also to the biophysical properties of the layer and the properties of the interface.

The aforementioned methodology used to make multi-layer composite biocatalysts can be used also to encapsulate different microorganisms by the same layer-by-layer technique. By using the same concept, the hydrophilic BC can be used for cell immobilization through the interaction between the BC nanofibrils and the cell wall mainly governed by hydrogen bridges. Likewise, the nano network of BC can be used for enzyme immobilization through hydrogen bridges. Similarly, two more biopolymeric layers containing different cells can be deposited over BC by the layer-by-layer method. The technique implies the production of a composite single cell biocatalyst, having as inner single cell, a cell 1 surrounded by nano BC fibrils and on it to attach a second layer of VS containing a microorganism 2. Moreover, on VS second layer can be adhered a third layer of ALG containing a microorganism 3. The BC/VS/ALG devices provide the possibility to produce three different products in the same bioreactor avoiding competition between cells of different cultures.

Biocatalyst attached on BC nano fibrils immobilized on the cell wall, gives the possibility to the cell to carry out bioprocesses that

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cannot otherwise be performed, due to cell does not produce this kind of enzyme. For example, *Saccharomycces cerevisiae* are not able to ferment starch and/or cellulose could carry out it, through cell outside activity, having extra cellular immobilized amylases and cellulases respectively. Similar cell outside biocatalyst activities could be obtained by combining other enzymes and microorganisms. The three layer enzymatic biocatalyst give the chance cell to carry out three bioprocesses having in each layer around cell encapsulated three different enzymes. Immobilized enzyme on BC nanofibrils network, is providing high concentration immobilized biocatalyst exposed to the medium that could give high conversion rate of substrates to products. This high contact between substrate and immobilized enzyme attributed to easy diffusion of substrates and products into aqueous solution through BC nanofibrils network is providing high enzyme turnover.

6.2. Cell inside activity bioprocessing by BC nanocomposite biocatalyst

The design is based on biopolymers of composites that could be hydrolyzed by cells. For that reason BC has attached immobilized enzyme (*i.e.*, amylase) and the second layer of VS to contain an encapsulated cellulase. The BC nano fibril will contains the bioactive molecule as nanowire, while the second layer of VS will have entrapped an electrolyte to play the role of electric tweezers (Hilmer and Strano, 2010). BC single fibrils have a length of about 80 nm and a width of about 4 nm (Ruka et al., 2014). Therefore, this could penetrate the cell wall. The single nano fibril of BC/VS composite will penetrate the cell wall by applying electric field (Fan et al., 2011) transfers in parallel way the nano composite biocatalyst inside the appropriate cell location, in order to liberate the bioactive molecule is contained as nano-wire in BC fibril of the composite. The liberation of bioactive molecule will be performed by the slow hydrolysis of cellulose and starch by cellulases and amylases respectively and may be it will not be necessary the use of photoactive technic. Then, glucose will be bioconverted on-demand by the cells.

The biocatalyst BC/VS nanocomposite for cell inside activity applications will be prepared by diffusion in BC bioactive molecule made by agitation of BC, in an aqueous solution of nanowire bioactive molecule. It will be followed by amylase enzyme immobilization on BC. The procedure is simple by immersing and stirring the BC matrix within solution containing amylase. Then, filtered and later the BC amylase composite will be immersed and stirred in VS containing cellulase and the electrolyte as electric tweezers. After filtration or centrifugation and drying the BC/VS nanocomposite containing both biocatalysts for cell inside activity has been prepared.

6.3. Electronics

Although BC as a biomaterial has been deeply studied for biomedical applications like drug delivery and tissue engineering, also others fields of research have been gaining relevance in the last years. Typical examples are the applications on the electronic/-magnetic fields using BC as a flexible matrix to develop materials with desired properties. In this way, ZnS/bacterial cellulose/epoxy resin (ZnS/BC/E56) nanocomposites with good transparency and flexibility were prepared. ZnS nanomaterials are applied as semiconductors and have excellent optical and photochemical properties. Besides, BC with epoxy components showed to have excellent flexibility and transparency. The authors reported a BC nanocomposite with enhanced transparency, thermo-optic stability and mechanical properties, presenting potential applications as a flexible optoelectronic biomaterial (Guan et al., 2016).

Another relevant approach was to use BC membranes for the development of Glucose fuel cells using glucose and oxygen to generate electric current as power supplier for medical implants (Zhang et al., 2015). The BC membranes containing silver nanoparticles operate as an electrode for Oxygen reduction showing higher current density (3.94 mA cm^{-2}) than the silver nanoparticles.

6.4. Medical field

Application of BC in the biomedical field have been gaining relevance in the last years (Silvestre et al., 2014). The combination of beta-glucanases (*i.e.*, cellulases) absence in humans and poor solubility in physiological media makes BC potentially useful for the development of membranes, patches or composites for medical applications like skin repair and tissue engineering. The physicochemical properties mentioned above plus the excellent biocompatibility of this biomaterial has opened the window for several applications in human health. For example, BC membranes are being used for wound healing and burn treatments (Petersen and Gatenholm, 2011). BC biocompatibility has been deeply studied by subcutaneous implantation in rats. The results were very promising since no fibrosis or encapsulation could be noticed around the BC implant. Besides, no macroscopic signs of inflammation, as redness, edema, or exudation, were observed. Moreover, cellular ingrown penetrates BC network forming a new integrated tissue with the biomaterial. Even vascularization and collagen synthesis took place. Finally, the total absence of chronic inflammatory responses allow to conclude that BC is totally biocompatible (Helenius et al., 2006). BC biostability have been also described when the matrix was used as a blood vessel replacement in sheep. The authors also described the good blood compatibility, minimal inflammatory potential, no blood cell lines changes compared to the control and functional stability after a three months the implantation experiment (Scherner et al., 2014). Nevertheless, it is important to mention that mammalians are not able to degrade cellulose because of lacking beta-glucanohydrolases acting over the cellulose chains. For some biomedical applications, this characteristic is an advantage (*e.g.* heart valves, meniscus) since the demand requirement of material with permanent stability and functionality. In other cases, the non-degradability might be a problem. Sometimes it is expected that new tissue displace the scaffold structure. However, it is interesting to note that bacterial cellulose seems to be integrated without any trouble into the hosted tissue as mentioned before (Scherner et al., 2014).

Cai and Kim reported a BC-(ethylene glycol) (PEG) composite prepared by immersing wet BC pellicle in PEG aqueous solution. The authors observed diffusion of PEG molecules into the BC network. Next, 3T3 fibroblasts were incubated with the scaffolds and differences of cell binding to the matrices between BC/PEG and plain BC were demonstrated. These novel BC/PEG scaffolds showed the potentiality to be used for wound dressing or tissue-engineering applications (Cai and Kim, 2010). Similar strategy was developed for BC in presence of alginate, and the BC/ALG hybrid matrix is able to increase 3 times the load of the anticancer drug doxorubicin and the device showed molecular controlled release of the load (Cacicedo et al., 2016).

Recently, BC membranes produced by *K. hansenii* were modified using inorganic salts by *ex-situ* method. Stereospecific nucleation of mesoporous hybrid microspheres composed of $CaCO_3$ and carrageenan was appended only to one side of BC films. The main objective was to develop an implantable a drug delivery device. The synthesis of the hybrid microparticles proceeds by self-assembly mechanism in the presence of calcium and contains tailorable amounts of the anticancer drug doxorubicin (Cacicedo et al., 2015).

In order to counteract the BC non-degradability in the human body some researchers have been developed chemical modifications on cellulose. Oxidized bacterial cellulose (OBC) was prepared

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in presence of nitrogen dioxide (NO_2). The oxidation reaction was controllable for tailoring the degree of degradation of BC (Peng et al., 2012). Moreover, BC with the integration of cellulases showed a controllable degradation process (Hu and Catchmark, 2011). Also, BC was modified by periodate oxidation to give rise to a biodegradable 2,3-dialdehyde bacterial cellulose (DABC) (Li et al., 2009).

6.5. Food Applications of BC

Since old times in the Southeast Asian culture BC was used for as source of healthy dietary fibers able to decrease the risks of some pathologies such as cardiovascular diseases, diabetes, diverticulitis and obesity (Shi et al., 2014). A typical example is the desert named *nata de coco* or *piña* (*i.e.*, coconut or pineapple cream) make by *K. xylinum* fermentation of coconut water plus fruits in a medium with high sucrose. Similarly, a static fermentation of black or green tea containing sucrose by cellulose-producers microbial consortium named Kombucha. After a period of two-weeks, the cellulose membrane on surface is removal and the liquid phase ready to drink (Shi et al., 2014). However, the Kombucha tea as a therapeutic infusion is still controversial because of the complex and undefined microbial consortium.

Additionally, BC can be used as thickening agent into pasty condiments, stabilizing agent in ice creams, gelling agent in some foods (*e.g.*, tofu) and stabilizing agent against temperature (*i.e.*, cocoa) (Shi et al., 2014).

Food packaging is another interesting application of native or modified BC as nano-reinforcement component or casting films. Addition of BC fibers increase thermoresistance, transparency and reduces diffusibility of gases and water sensitivity of starch and PLA composite films (Rhim et al., 2013).

6.6. Other Bacterial cellulose applications

Cellulose, as fibrils or films, has been proposed as reinforcement material to make blends, composites and nanocomposites with potential application in many fields. Hybrid films of BC with hydrophilic and hydrophobic polymers from polyvinylchloride (PVC), latex to polyvinyl alcohol displayed several physicochemical advantages recently reviewed (Siró and Plackett, 2010). Food packaging is an interesting application of native or modified BC as nanoreinforcement component of films. Addition of BC fibers increase thermoresistance, transparency and reduces diffusibility of gases and water sensitivity of starch and PLA composite films (Rhim et al., 2013). Also, considering gelling and thickening properties of BC, it is widely appreciated in the food industry. Two types of chemically modified plant celluloses such as carboxymethyl cellulose (CMC) and hydroxypropyl methylcellulose (HPMC) are used as additives in foods. However, BC showed better stabilizing properties in oil/water emulsions independent of environmental stresses such as pH and temperature and compared to HPMC and CMC (Paximada et al., 2016a). The mechanisms postulated by the authors was the adsorption of the oil droplets into the fibrils surface forming strong network. Similarly, emulsions made of olive oil-whey protein isolate showed superior stabilization by the addition of BC as thickener used also at lower concentrations compared to locust bean gum and xanthan gum (Paximada et al., 2016b).

Addition of BC fibers to bagasse-cement composites improves physical and mechanical properties of the composite with the advantage of being biodegradable and environmentally friendly (Mohammadkazemi et al., 2015b).

Also, BC films can be applied for the restoration of old paper-made materials, a powerful tool for preserving ancient documents and manuscripts in libraries and museums. The lining method consist in apply a reinforcing BC layer over the surface document bringing high stability over the time (Santos et al., 2015).

7. Conclusions

BC is a promissory material for the development of biotechnological devices covering many different fields. The production of BC from different microorganisms is being optimized and the reduction of production costs, and scale up optimization are a technological prerequisites to be adopted at large scale. Meanwhile, specific applications of BC in nanoelectronics, bioprocesses (*e.g.* biotransformations, cells production) and nanomedicine are very promising areas for the development of new era of Green Chemistry products.

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