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## New insights into bacterial cellulose materials: production and modification strategies

Manuel Horue<sup>1</sup>; Maximiliano L. Cacicedo<sup>1</sup>; Guillermo R. Castro<sup>1\*</sup>.

<sup>1</sup>Laboratorio de Nanobiomateriales, CINDEFI (Universidad Nacional de La Plata - CONICET, CCT La Plata), Calle 47 y 115. C.P. 1900 La Plata, Argentina.

\*Corresponding author: E-mail address: [grcastro@quimica.unlp.edu.ar](mailto:grcastro@quimica.unlp.edu.ar)

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#### ABSTRACT

*Komagataeibacter xylinus* cultures produced a high amount of bacterial cellulose (BC), which structure consists in a nanoporous network of interlaced fibers. When the culture is performed under static experimental conditions, a membrane with characteristics of highly hydrated hydrogel and good mechanical properties is obtained with promissory applications in the biomedical field. Bacterial cellulose films can be used for many application such as dermal dressing, scaffolds for tissue regeneration and even as a controlled drug release system. Besides, stirred cultures of *K. xylinus* produced amorphous cellulose structures dispersed in the medium with physical and mechanical characteristics different from the membrane. In addition, new properties of BC can be obtained or added if the hydrogel is mixed with other compounds or modified post-purification using both organic and inorganic compounds.

### Introduction

Hydrogels are networks shaped by hydrophilic polymer chains which exhibits the ability to swell and retain a significant high fraction of water in their structure, but do not dissolve in it.<sup>1</sup> The advantage of absorbing and retain high amount of water by hydrogels gives softness, elastic consistency and superficial similarity to living tissue. In addition, hydrogels are permeable to small molecules such as oxygen, nutrients and metabolites.<sup>2</sup>

Particularly, Bacterial cellulose (BC) is classified as a hydrogel composed of polymer chains are made up of  $\beta$ -D-glucopyranose monomers linked by beta (1-4) glycosidic linkages.<sup>3</sup> Hydrogen bridges, either inter-chain or intra-chain, which exist due to the high amount of hydroxyl groups of the sugar skeleton hold the chains together and allow them to associate and entangle each other through a self-assembly process, leading to a three-dimensional structure.<sup>4</sup> Particularly, cellulose produced by bacteria has many advantages over cellulose obtained from vegetable sources. The cellulosic material obtained from plants has complex and heterogeneous structures. Plant cellulose is intimately associated with other polymers such as lignin and hemicellulose building complex morphologies.<sup>5</sup> These accessory polymers have specific functions in the physiology of plants. However, for biomedical purposes the plant cellulosic material is required to be intensively purified. In addition, purification processes for vegetable cellulose involve complex and quite expensive mechanisms. For example, mechanical treatments and chemical pre-treatments are often used, which consume a lot of electrical energy

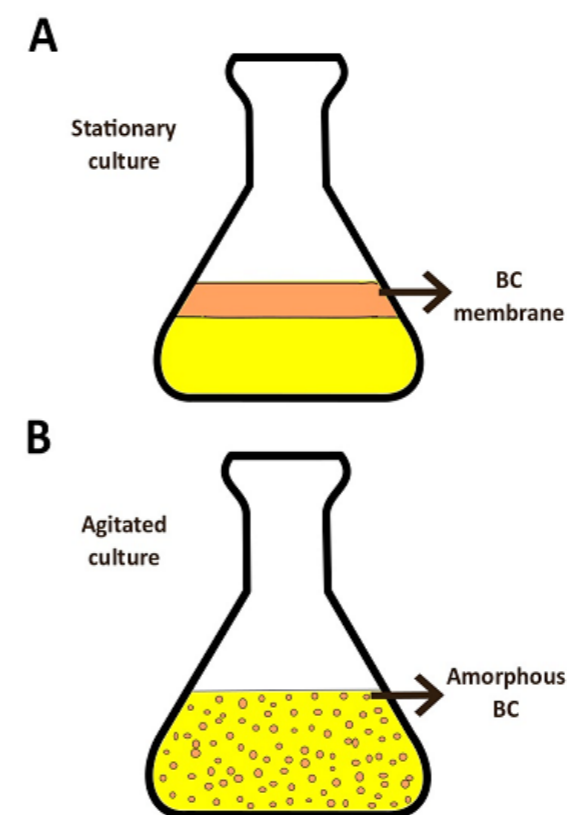
and high concentrations of acids and bases, both environmentally pollutants.<sup>5</sup> Meanwhile, bacterial cellulose is produced in a pure form, being the purification process simple, economical and friendly with the environment.<sup>3</sup>

The most common BC-producing microorganisms are members of the *Acetobacteriaceae* family, particularly those belonging to the genus *Komagataeibacter* (formerly called *Gluconacetobacter*). They are Gram-negative, strict aerobic bacteria.<sup>3</sup> The production of BC in liquid media under static culture conditions shows an extensive membrane covering the air/liquid interface of the culture. However, BC is synthesized dispersed in the liquid medium if the liquid culture is stirred during the bacterial growth. The resulting BC structures are generally irregular spheres and/or suspended fibers. The choice of any of these cultivation strategies depends on biopolymer application.<sup>6</sup>

One of the most interesting characteristics of BC is that it can be modified in different ways through environmentally friendly techniques. Unlike other polymeric materials, the processes of production and modification of BC are considered Green Chemistry procedures because of they can be carried out without the use of organic solvents and/or toxic molecules and without any other compound that contaminates the environment. The main strategies of BC modification can be divided into two types: *in-situ* modifications where exogenous materials, such as polymers, are added to the culture medium. The cellulose fibers self-assembled as they are being synthesized, and the exogenous material is incorporated to the network by interacting with the BC fibers. At the end of the process, a hybrid BC

fiber network with physicochemical characteristics different from those found in a native BC network is obtained. These new characteristics are contributed by the exogenous material and by its interaction within the cellulose fibers and involve intimate modifications in the structure of the BC. On the other hand, *ex situ* modifications consist of all those modifications that are made to the BC after its production and purification process.<sup>7</sup>

The aims of the work are to review the main properties of BC and the "Green strategies" of cellulose modifications in static and agitated cultures to obtain different materials with novel properties (e.g. BC membranes and amorphous BC).



**Figure 1** - Cartoon of bacterial cellulose synthesized in (A) static and (B) agitated cultures.

### BC production

The culture of microbial species of *Komagataeibacter* genus and the production of bacterial cellulose (BC) constitute a biotechnological process, which depends on many intrinsic factors such as the bacterial strain, culture medium (mainly carbon and nitrogen sources) and extrinsic factors such as environmental (e.g. temperature, pH, etc.).

Specifically, *K. xylinus* species can use diverse carbon sources from monosaccharides (5 and 6 carbons length chain) to oligosaccharides and polymers (e.g. starch), or other molecules such as alcohols and organic acids.<sup>8,9</sup> However, the BC yield will depend on the metabolic pathway of each carbon source. For example, supplementation with ethanol to media containing glucose results in an

increase in cellulose productivity, which could be partially attributed to the increase of cellular membrane permeability.<sup>10,11</sup> In a recent work, the authors reported an increase in more than 500 BC yield by supplementing with 1% of rapeseed oil to Hestrin-Schramm medium (containing 2% glucose and 0.115% citric acid as main carbon sources). The authors also claimed an increase of 285% BC thicker and correlated with high tensile strength compared to the control without oil supplementation.<sup>12</sup>

Regarding the nitrogen sources, the highest yields are given for the combination of yeast extract and peptone in the culture medium.<sup>11</sup> On the other hand, the optimum pH and temperature for cellulose production using *K. xylinus* was found in the range of 5.0-7.0 and 28-30°C, respectively.<sup>13,14</sup>

*K. rhaeticus* isolated from kombucha tea was able to produce BC in a Hestrin and Schramm medium partially or totally supplemented with sugar cane molasses, reducing the BC production costs up to 20%, a critical point for BC production at large scale.<sup>15</sup> Following the same line of work, *Gluconacetobacter sucrofermentans* B-11267 was able to synthesize BC in media containing acid food by-products such as cheese whey at pH= 4.96 and thin stillage at pH= 3.95, 5.0 and 6.0. The BC production showed an increase of 2.5 to 3-times compared with bacterial cultures in Hestrin and Schramm medium under similar experimental conditions. X-ray analyses of BC films showed a change in the BC microfibril width and crystallinity but without changing its chemical structure.<sup>16</sup>

The biochemical process of BC synthesis consists of three main steps: (i) polymerization of glucose residues in the  $\beta$ -1-4 glucan chains, (ii) extracellular secretion of linear chains through pores or terminal complexes of the bacterial cell (CTs) with 3.5 nm in diameter and (iii) organization and crystallization of glucan chains in hierarchical arrangements due to hydrogen bridge interactions and van der Waals forces.<sup>6</sup> The nascent chains form 1.5 nm sub-fibers wide which in turn are assembled into nanofibers of 2-4 nm in diameter. Then, these nanostructures are associated in more complex structures in the form of films of 40-60 nm wide and with a thickness of 3-8 nm that can intermingle and entangle forming an exceptional 3D network that gives rise to the membranes or films (Figure 2).<sup>3</sup> BC in these membranes, the crystalline structure is higher than 70%.<sup>17</sup>

### Static cultures

Static cultivation of *K. xylinus* in liquid media (Figure 1A) produces floating cellulose membrane at the interface liquid-air that helps the bacteria to have large availability of oxygen.<sup>18</sup> In addition, the BC membrane protect the bacteria from other microorganisms in the environment and works as a physical barrier against UV radiation and redox processes. Also, BC membrane increases the ability to colonize other places and maintains a hygroscopic environment avoiding periods of dehydration and lack of moisture.<sup>19,20</sup>

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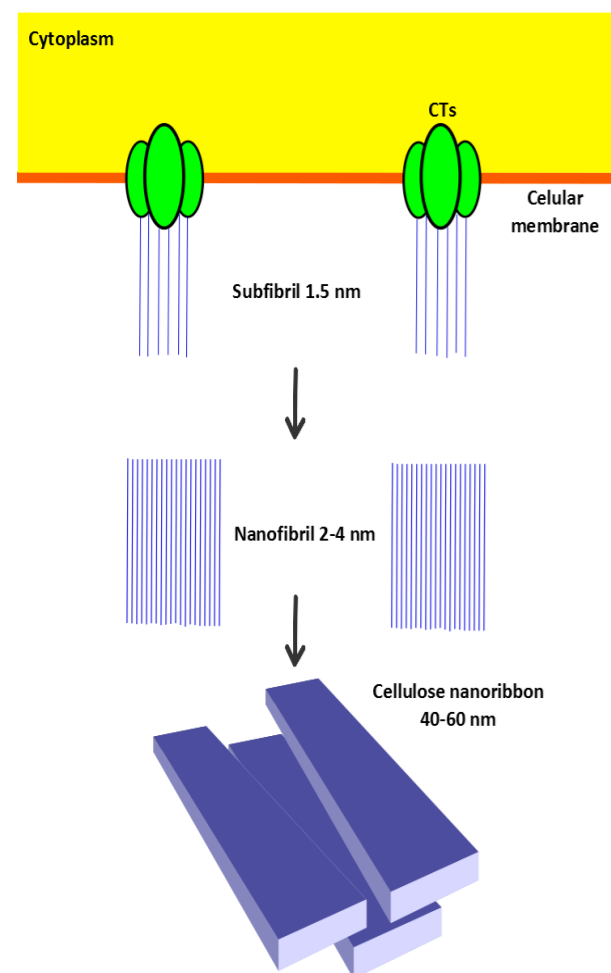
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### Agitated cultures

When *K. xylinus* is cultivated under constant agitation it is not possible to obtain a membrane as in the static culture, but cellulose synthesis is observed as small spheres and/or amorphous agglomerations (Figure 1B). The BC yields in these types of cultures are lower than those of the static culture because of the growth of non-BC producing bacterial cells which are competing and consuming the substrates. The decrease of BC yields was attributed to the accumulation of mutations that damage the machinery responsible for polymerizing glucose.<sup>21-23</sup>



**Figure 2** - Bacterial cellulose biosynthetic and self assembly representation of network (modified from Cacicedo *et al.*, 2016).

Additionally, a large amount of glucose is converted to other molecules such as gluconic and keto-gluconic acids that are released into the medium. The deviation of carbon source utilization by alternative metabolic pathways leads to the synthesis of other metabolites causes a detriment in the cellulose production.<sup>22,23</sup> A comparative study reported at least 3-times lower BC yield, from 28 g/L and 9 g/L of BC obtained in static and agitated cultures respectively.<sup>23</sup> Also, strong changes of BC morphological and crystalline structure under static and agitated cultivation were reported.<sup>22,23</sup> In both studies, the reticulated structure of fibers is maintained in both culture methodologies but with subtle

changes, since the fibers presented a superior curvature in the agitated cultures and they were more entangled among themselves giving rise to a denser reticulated matrix. These changes are supposed to be related to variations at the BC microstructure level, such as the degree of polymerization, the crystallinity and the relationship between the  $I\alpha$  and  $I\beta$  cellulose allomorphisms.<sup>22,23</sup> According to their reports both the crystallinity and the size of the crystals are diminished under conditions of agitated cultivation. The reports also found that the BC  $I\alpha$  allomorphism decreases and the more stable BC  $I\beta$  increases, concomitantly with the degree of polymerization of the glucose chains, which is reduced in compared with the static culture.

### BC properties

Bacterial cellulose matrices possess unique properties, such as:

- Ability to retain a large amount of water, up to 99% of its content, which exceeds cellulose obtained from plant sources. A physical type hydrogel is thus formed.<sup>5</sup>
- High crystallinity and high degree of polymerization that confer excellent mechanical properties, superior to vegetable cellulose. The tensile strength of the BC is usually between 200 and 300 MPa, and its Young's modulus is between 15 and 35 GPa.<sup>24</sup>
- High thermal resistance due to its high degree of purity and crystallinity. This property is very important for biomedical applications because allows the biomaterial to be thermally sterilized.<sup>25</sup>
- Biodegradability that classifies BC as a green material.<sup>26</sup> BC is not be able to be degraded by mammalian cells because of lacking 1,4- $\beta$ -D-gluco-hydrolase activities. Meanwhile, BC can be hydrolyzed by several microorganisms in the human gut and in the environment with the ability of express enzymes capable of break 1,4- $\beta$ -D-glycosidic linkages.<sup>27</sup>
- Excellent biocompatibility. *In vivo* studies of subcutaneous implantation of membranes in rats showed that they do not produce fibrosis or granulomas after 12 weeks, which shows that there is no reaction to foreign bodies. In addition, there was no redness, swelling or edema around the site of implantation site.<sup>28</sup>

• The BC membranes are asymmetric. The surface of BC in contact with the air are very closed polymeric network with narrow size porosity. Meanwhile, the BC surface in contact with the liquid media displays pending cellulose chains because of the bacterial cell synthesis and able to be easily tailored applying different strategies.<sup>3</sup>

Another relevant aspect related to the production of membranes is the versatility of BC production. The shape and thickness can be easily controlled by varying the mold type of the reactors and the cultivation time.<sup>3,29</sup>

The unique properties of the BC have inspired its use in numerous commercial products, including strips, headphone membranes, high quality paper, dietetic foods and textiles. However, the most promising properties of BC are

found in the biomedical field for multiple purposes such wound dressing, synthetic skin, scaffolds for tissue engineering, artificial blood vessels, controlled drug release systems and dental implants.<sup>3,5,29</sup>

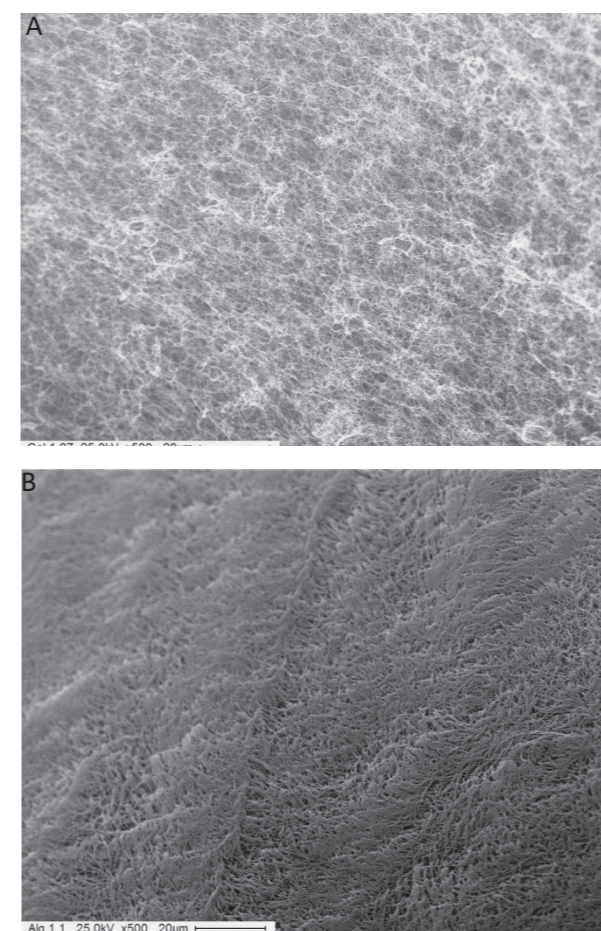
### Modification of BC

Although, the choice of BC production methodology possesses the advantage of tailoring its properties creating new ones that native BC does not have. The simplest procedure to tailor BC is to incorporate a non-degradable molecules/structures in the culture creating a hybrid BC structures. Exogenous material can be polymers, nanoparticles, metals, metal oxides, clays and solid particles of macrometric size. There are two main ways of convert native BC into hybrid materials using Green Chemistry techniques which will be described below.

### In-situ modification

In this procedure the reinforcing material is added to the culture medium at the beginning or during the biosynthetic process.

This technique is often accompanied by significant changes in the BC structure, higher than other strategies, giving rise to compounds with physicochemical characteristics and distinctive properties (Figure 3).<sup>7</sup>



**Figure 3** - Scanning electron microscopies of native bacterial cellulose (A) and Alginate-Bacterial cellulose composite (B) <sup>7</sup>

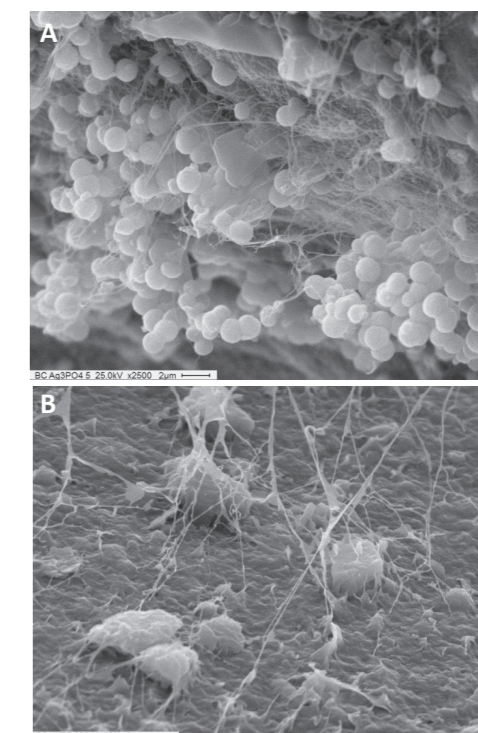
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Several BC compounds have been generated from this type of methodology by the addition of different compounds such as polyvinyl alcohol, graphene oxide, carboxymethylcellulose, alginate and even an extract obtained from *aloe vera*, obtaining characteristics and properties very different from those of the materials separately.<sup>7,30-33</sup>

### Ex-situ modification

In this method, the BC can grow conventionally, and after purified the membranes are mixed with the reinforcing material. This technique is very versatile and simple, and the most important factor when choosing it is that the original structure of the BC remains almost unchanged because the exogenous material added does not interfere with the assembly of microfibrils in *de novo* synthesis. The integration of the material depends on its size and chemical nature, so only submicrometric and nanometric materials can interpenetrate the network in a homogeneous way because they fit in all its pores; and non-polar materials would not be combined with the membrane.<sup>7</sup>

This strategy has successfully incorporated numerous compounds on BC matrices, including chitosan, silica, silk, silver nanoparticles, phosphate microparticles and even clay minerals.<sup>34-41</sup> The hybrid materials formed showed an alteration in the properties in relation to the native BC (Figure 4).



**Figure 4** - Scanning electron microscopies of Ex-situ bacterial cellulose modified with silver phosphate (A) and Montmorillonite (B) microparticles respectively.<sup>40, 41</sup>



## Conclusions

The cultivation of bacterial species of the *Komagataei-bacter* genus and the manipulation of different parameters of the biotechnological production process allows the obtention BC with different morphologies, dimensions, and topologies according to the application that is wanted to be given to the biopolymer. If the culture is performed statically, a membrane is produced at the interface between the liquid and the culture medium. The highly hydrated character, the good mechanical properties and the biocompatibility make these membranes a promising material in biomedicine, where it has diverse applications. However, if the culture is stirred during the incubation period the cellulose produced has a morphology of spheres and/or fibers dispersed in the culture medium. The versatility with which the BC is produced is one of the main causes of its wide range of applications. Most of the research works carried out have sought to study the applications of cellulose produced in the form of a membrane by means of static cultures. Besides, there is a vacancy in the study of agitated cellulose cultures and their biophysical properties under these experimental conditions.

On the other hand, the two modification strategies of the BC membranes allow to obtain hybrid materials by soft techniques with novel and optimized properties for the different applications. *Ex-situ* modification has proven to be a versatile, simple and very useful modification method when the exogenous component is toxic or unstable to or in the bacterial culture or in the presence of the growing membrane. In contrast, *in-situ* modifications are more complex processes and the added material must be compatible and able to remain in solution or suspension in the culture medium during BC membrane growth. However, the degree of modification on the cellulose membranes by *in-situ* is higher and the presence of the external component in the hybrid material increased.

Finally, bacterial cellulose continues to be present as a valuable biomaterial for several applications. Even though in recent years has increased the research in the BC field, it is relevant to mention that there are still some vacant areas of BC production under diverse experimental conditions to be explored. Consequently, it is essential to explore how these changes could affect the structure, composition and biophysical properties of diverse composite BC materials.

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