PRODUCTION OF BACTERIAL NANOCELLULOSE INALTERNATIVE CULTURE MEDIA UNDER STATIC AND DYNAMIC CONDITIONS

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***Abstract* — Bacterial nanocellulose (BNC) was produced in three different culture media: the standard HS medium, analytical glycerol/corn steep liquor (AG-CSL) medium and glycerol remaining from biodiesel production/corn steep liquor (BG-CSL) medium. Both static and agitated conditions were assessed, and the effects of the culture medium, agitation speed and cultivation time used were evaluated on BNC production and selected products characteristics (morphology, specific surface area and crystallinity). Results showed that the cheaper alternative culture media assayed led to increased production of BNC, and that agitation speed modulation allowed producing either BNC pellicles or BNC pellets with varying size, shape, crystallinity and cellulose nanofibers packing.**

***Keywords* — microbial cellulose; glycerol; corn steep liquor; static vs agitated culture; characterization.**

I. INTRODUCTION

Bacterial nanocellulose (BNC), chemically identical to plant-based cellulose, is synthesized as an extracellular primary metabolite by various genera of aerobic bacteria (*e.g. Acetobacter*, *Azotobacter*, *Agrobacterium*, *etc*). *Gluconacetobacter xylinus* (formerly, *Acetobacter xylinum*, now syn. *Komagataeibacter xylinus*) grouped within the acetic acid bacteria, is the most widely used producer of BNC. Distinguishing properties of microbial cellulose include its high purity (bacteria produce it free of hemicellulose, lignin and pectin contained in plants, thus no economically and environmentally costly purification processes are required), and its production as a fine web of fibers of micrometric length and nanometric section. BNC is characterized by remarkable mechanical properties, high water holding capacity, moldability, biodegradability in varying ecosystems, and excellent biological affinity. Based on these characteristics, in the last years several applications of BNC have been proposed including reinforcement of nanocomposite materials, non-resorbable 3D cell scaffolds, wound dressings, filtration membranes, cosmetic masks, paper additives, hygiene absorption products, and rheology modifiers, among others (Charreau *et al.*, 2020).

BNC can be produced in both static and agitated conditions. Due to the strictly aerobic character of the bacteria used, in static culture BNC is obtained as a highly hydrated pellicle formed at the air-liquid interface of the fermentation vial, which is constituted by a three-dimensional network of cellulose nanoribbons. Production of BNC in static culture is simple and requires low technology investment, but it relays on large surfaces to overcome the O_2 diffusion limitation, and long fermentation times are usually required (Foresti *et al.*, 2015). On the other hand, in agitated culture, BNC nanoribbons organize into discrete particles with varying morphologies (*e.g.* stellate and dispersed fibrous strands, regular spherical shapes, spherical bodies with surface ramifications, cocoon like structures, etc.). Previous studies have reported the effect of different factors on the morphology and size of the BNC particles obtained in dynamic culture including strain (Bi *et al*., 2014; Singhsa *et al*., 2018), medium composition (Algar *et al*., 2015, Brandes *et al*., 2018, Hu *et al*., 2013), fermentation time (Zhu *et al*., 2011), and agitation speed (Hu and Catchmark, 2010; Zhu *et al.* 2011; Żywicka *et al.*, 2015). In agitated systems strong mechanical agitation prevents heterogeneity of the culture medium, and controls and scale up can be more easily implemented. On the other hand, the production of BNC in agitated systems requires higher power supply, and greater and more uniform aeration of the medium volume in stirred conditions has sometimes been associated with the appearance of cellulose-nonproducing mutants (the latter due to effective access to oxygen with no requirement of BNC membrane formation at the air-medium interface). BNC particles obtained in dynamic culture have proved useful for enzyme immobilization, injectable orthopedic scaffolds, drug incorporation, and dye and heavy metal ions removal (Brandes *et al*., 2018; Hu *et al*., 2013; Meng *et al*., 2019; Zhu *et al.*, 2011).

Independently of whether fermentation medium is agitated or not, at laboratory scale BNC is conventionally produced using the Hestrin & Schramm medium (HS, Hestrin and Schramm, 1954), in which the carbon source is glucose and yeast extract and peptone are added as micronutrient, vitamin and nitrogen sources. However, the cost of the HS standard medium often imposes a limitation for scale up. In this context, in the last years several

studies have shown the suitability of alternative cheaper raw materials for BNC production in media formulated with agroindustrial wastes and by-products. Among them, beet and sugar cane molasses, soya bean whey, cheese whey, sulfite pulping liquor, glycerol from biodiesel, wine residues, banana peel extract, maple syrup, pineapple peel juice, and corn steep liquor (a by-product from the corn-steeping process rich in organic nitrogen and usually used for animal feed and antibiotic production medium purposes), are just some examples of C or/and N sources which have been evaluated for BNC production in static culture (Fernández Corujo *et al.*, 2016).

In the current contribution BNC was produced in both static and agitated conditions in three different culture media, *i.e.* standard HS medium, analytical glycerol/corn steep liquor medium (AG-CSL), and glycerol remaining from biodiesel production/corn steep liquor medium (BG-CSL). The effect of agitation speed and cultivation time on BNC production (g L^{-1}) were both evaluated, and products were characterized in terms of morphology, specific surface area and crystallinity. To the best of the authors' knowledge no previous contribution has focused on the production and characterization of BNC pellicles and particles obtained in the alternative fermentation media described.

II. METHODS

A. Materials

The standard Hestrin and Schramm (HS) medium involved anhydrous dextrose (Biopack), peptone (Britania), yeast extract (Britania), disodium phosphate (Anedra) and citric acid (Merck). Chosen alternative culture media implied the use of analytical glycerol (AG, Stanton), glycerol remaining from biodiesel production (BG, provided by a local biodiesel production facility with a purity of 70 % (w/v)), and corn steep liquor (CSL, Ingredion). NaOH (Biopack) was used to eliminate bacterial cells during BNC purification.

B. BNC production

BNC was produced in both static and agitated culture using *Gluconacetabacter xylinus* NRRL B-42. In all cases the inocula of *G. xylinus* were cultured in static conditions for 48 h at 28ºC in 100 mL Erlenmeyers flasks containing 20 mL of HS medium (anhydrous dextrose 2.0 % (w/v), peptone 0.5 % (w/v), yeast extract 0.5 % (w/v), disodium phosphate 0.27 % (w/v) and citric acid 0.115 % (w/v)). After this interval, flasks were shaken vigorously in a vortex mixer to remove bacteria from the pellicles. For BNC production, 1 % (v/v) inocula were transferred to 250 mL Erlenmeyers containing 100 mL of each fermentation medium. Three culture media were used: standard HS medium, analytical glycerol (4% w/v)/corn steep liquor (4% w/v) medium (AG-CSL), and glycerol remaining from biodiesel production (4% w/v)/corn steep liquor medium (4% w/v) (BG-CSL). Media were incubated in a thermostatic bath (Dubnoff Digital, Vicking, Argentina) at a constant temperature of 28ºC, in both static (*i.e.* 0 rpm) and agitated conditions (90, 130, and 150 rpm, using a linear water bath shaker), during 48 h and 72 h. Fermentations were run in duplicate, reaching a total number of 48 assays (Table 1). BNC membranes (static culture) and pellets (agitated culture) formed were harvested, thoroughly rinsed with distilled water to remove the culture medium, and treated with NaOH solution 2 % (w/v) at 100ºC for 1 h to eliminate the bacterial cells. Samples were recovered by filtration and rinsed with distilled water till neutrality of the wash waters. Finally they were either dried at 105°C until constant weight (for BNC production quantification), or freeze-dried (for certain characterization assays in which retention of their original 3D structure was required) during 48h in a Labconco FreeZone 2.5L Benchtop Freeze Dryer operated at −50°C and 0.12 mbar. BNC production was expressed as the dry weight of cellulose per liter of the original medium (g L^{-1}).

C. BNC characterization

Optical microscopy: Samples of BNC assemblies obtained in static and agitated culture were purified as previously described and observed in the hydrated state in a Digital Microscope (Microscopio Digital Electronic Magnifier USB; software for image treatment: Cooling-Tech Microscope).

Field emission scanning electron microscopy (FESEM): Freeze-dried BNC assemblies were observed in a scanning electron microscope Zeiss Supra 40 (Dresden, Germany) with a field emission gun operated at 3 kV.

X-ray diffraction analysis (XRD): Freeze-dried BNC assemblies were analyzed in a Rigaku D/Max-C Wide Angle automated X-ray diffractometer with vertical goniometer (Cu/Kα radiation source 0.154 nm, 40 kV, 30 mA). Diffractograms were recorded at 0.6°/min in the 10-50° 2θ intervals, at a step size of 0.02º. The crystallinity index (CI) of BNC samples was estimated by use of Segal's empirical equation (Eq. 1, Segal *et al.*, 1959), where I_{002} corresponds to the maximum intensity of the 002 lattice diffraction and accounts for both crystalline and amorphous material, and I_{am} is the intensity at 2θ=18º and stands for amorphous material only.

$$CI = (I_{002} - I_{am})/ I_{002} \cdot 100. \quad (1)$$

Brunauer-Emmett-Teller (BET) surface area analysis: The specific surface area (SSA) of freeze-dried BNC samples was determined by nitrogen adsorption at 77 K in a Micromeritics ASAP 2020 instrument. SSA values were determined using the BET equation (Brunauer *et al.*, 1938). Before the measurement, samples were degassed to remove any adsorbed water molecules.

III. RESULTS

A. BNC production

BNC was produced in both static and agitated conditions using the three culture media previously described (Table 1). BNC production values are summarized in Fig. 1.

Results included in Fig. 1 evidence that, independently of the fermentation time and agitation speed used, the media formulated with CSL and either analytical glycerol or residual glycerol from biodiesel

Table 1. Codification of the assays performed. Medium composition/agitation speed/fermentation time

	Medium composition					
Agitation speed (rpm)	**HS**	**HS**	**AG-CSL**	**AG-CSL**	**BG-CSL**	**BG-CSL**
0	HS/0/48	HS/0/72	AG-CSL/0/48	AG-CSL/0/72	BG-CSL/0/48	BG-CSL/0/72
90	HS/90/48	HS/90/72	AG-CSL/90/48	AG-CSL/90/72	BG-CSL/90/48	BG-CSL/90/72
130	HS/130/48	HS/130/72	AG-CSL/130/48	AG-CSL/130/72	BG-CSL/130/48	BG-CSL/130/72
150	HS/150/48	HS/150/72	AG-CSL/150/48	AG-CSL/150/72	BG-CSL/150/48	BG-CSL/150/72
Time (h)	48	72	48	72	48	72

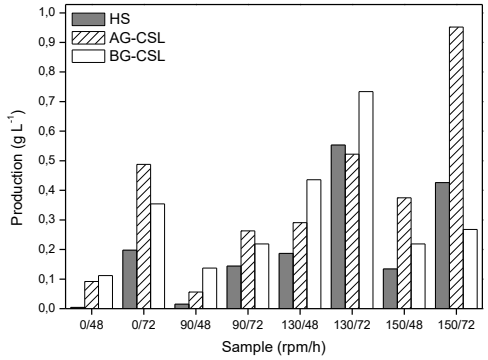

Figure 1. Effect of agitation speed, culture media and fermentation time on BNC production.

production generally led to BNC production values significantly higher than those attained in the standard HS media. The alternative culture media herein used contained an optimized glycerol and CSL concentration, which accounted for a carbon content twice that of the typical HS medium. The importance of considering the C source concentration when comparing different pre-optimized BNC culture media with conventional HS medium has previously been described (Ruka *et al.*, 2012). By the way, other contributions dealing with BNC production using *G. xylinus* in the standard HS medium (glucose as C source) and HS medium modified by replacing the C source with glycerol at equal C source content, have previously reported a much higher BNC production when using analytical glycerol instead of glucose (Keshk and Sameshima, 2005; Mikkelsen *et al.*, 2009; Tabaii and Emtiazi, 2015). Better results obtained when using glycerol instead of glucose as the main carbon source have been frequently related to the effect of pH in BNC production. Optimum pH for BNC production depends on the bacterial strain used, being usually in the 4.0-7.0 interval for *Acetobacter xylinum* (Reiniati *et al*., 2017). The growth of the microorganism is adversely affected at lower pH, with a direct effect on BNC production (Zahan *et al.*, 2015). Important pH reductions in media formulated with glucose as the sole C source have been associated with the formation of gluconic acid (Zhong *et al.*, 2013). Contrarily, glycerol has been reported to switch its path from the pentose cycle to the Krebs cycle without accumulation of gluconic acid, resulting in lower pH fluctuation during the fermentation process and significantly greater BNC productions (Thorat and Dastager, 2018; Zhong *et al.*, 2013). Moreover, the CSL herein used must have also played a role in the improvement of BNC production in AG-CSL and BG-CSL media in comparison to the traditional HS culture. Being a well-stablished low-cost source of proteins and vitamins, this by-product of the corn wet-milling process has also been reported to contribute to high BNC production by maintaining the culture pH value within the optimum range for BNC synthesis (Jung *et al*., 2010). Actually, the capacity of CSL for maintaining the pH within the optimal range for BNC production without the need of using a pH electrode or pH controller (and thus avoiding the practical measurement difficulties associated with BC attachment to the electrodes and viscosity increment during cultivation) has long been demonstrated in shake-flasks, stirred-tank reactors and airlift reactors (Noro *et al*., 2004).

With regards to BNC production attained with either analytical or residual glycerol, their relative values showed to be dependent on the particular agitation speed and culture time chosen (Fig. 1). Crude glycerol is a by-product of biodiesel production which can be either converted into pure glycerol for several industrial applications, or otherwise used with little or no purification for the production of high-value chemical products such as organic acids, polyols and biopolymers by chemical or biological routes (Dobson *et al*., 2012; Garlapati *et al.*, 2016). In particular, crude glycerol has been previously evaluated for the production of BNC in agitated (Adnan, 2015) and static culture (Gayathri and Srinikethan, 2018; Jin *et al*., 2019; Vázquez *et al*. 2013). The few available reports comparing BNC production using analytical versus crude glycerol as carbon source have generally reported higher production with the analytical polyol, the previous often associated with potential effects of inhibitory compounds present in crude glycerol (Carreira *et al*., 2011; Jin *et al*., 2019; Vázquez *et al*. 2013).

In regard to the effect of culture time, for most systems assayed not only production but also productivity (*i.e.* production/elapsed time, g L^{-1} day^{-1}) was found to increase from 48h to 72h. Similar results were previously obtained within the 48h-72h interval in both stationary and agitated conditions when studying the time course of BNC synthesis with *G. xilynus* in HS medium (Czaja *et al*., 2004) and in modified HS medium containing analytical glycerol as C source (Kim *et al*., 2006).

Concerning the effect of agitation speed, under the conditions set, the systems agitated at the highest rates *(i.e.* 130 and 150 rpm) showed larger BNC production values than those attained in static culture and also in systems agitated at 90 rpm. Agitated culture has often been associated with lower BNC productions than static

culture, a phenomenon often attributed to the appearance of non-producing mutants in agitated systems (Aydın and Aksoy, 2014; Krystynowicz *et al*., 2002). On the other hand, other authors have reported the increase of BNC production in agitated systems (Singhsa *et al*., 2018), with some of them also finding maximum BNC production values at 150-170 rpm (Mohite *et al*., 2012; Son *et al*., 2002). Results have been attributed to improved aeration achieved at high stirring rates which might have resulted in larger air–liquid surface area (Mohite *et al*., 2012), higher oxygen availability and thus higher BNC production.

B. BNC morphology

Figure 2 collects representative photographs of the BNC assemblies obtained in each of the systems evaluated. As expected, whereas with sufficient fermentation time static culture led to cellulose pellicles of increasing thickness grown at the air-liquid interface, agitated culture resulted in discrete pellets with varying shapes and sizes. The speed of agitation is known to affect the structure and size of the BNC pellets obtained in stirred media, potentially due to different shear forces present at the surface of the forming particles (Gu and Catchmark, 2012). In the systems herein assayed, when the lowest agitation speed was used (*i.e.* 90 rpm) spherical pellets of *ca.* 1 cm were obtained in the HS medium, whereas in glycerol-containing media less defined structures were produced. On the other hand, in all culture media assayed the increase of stirring speed led to much smaller particles (Fig. 2), in agreement with previous contributions which associated increasing rotational speeds during BNC culturing with remarkably decreased particle sizes (Hu and Catchmark, 2010; Son *et al*., 2002). Closer views of some of the most abundant pellet morphologies obtained after 72 h of fermentation with the highest stirring speeds assayed are shown in Fig. 3. Ellipsoidal, reniform and fiber-like structures were found, and particles with none, single and multiple branched and unbranched projections were observed.

Figure 4 collects some FESEM micrographies of the BNC assemblies obtained. As it is shown, in all cases they are constituted by an entangled web of twisting cellulose fibrils with micrometric lengths and nanometric widths (≈ 20-80 nm) and thicknesses.

C. BNC crystallinity

Based on the generally highest BNC production achieved in media containing analytical glycerol and CSL, BNC assemblies obtained at 72h in the AG-CSL medium were the ones chosen for assaying the effect of stirring speed on BNC crystal structure and relative crystallinity. Diffractograms are shown in Fig. 5. In all cases, the typical Cellulose I structure of BNC with three well-defined diffraction peaks centered at 2θ = 14.4° (101), 16.7° (10-1) and 22.6° (002) was observed (Johnson *et al*., 2010). Less

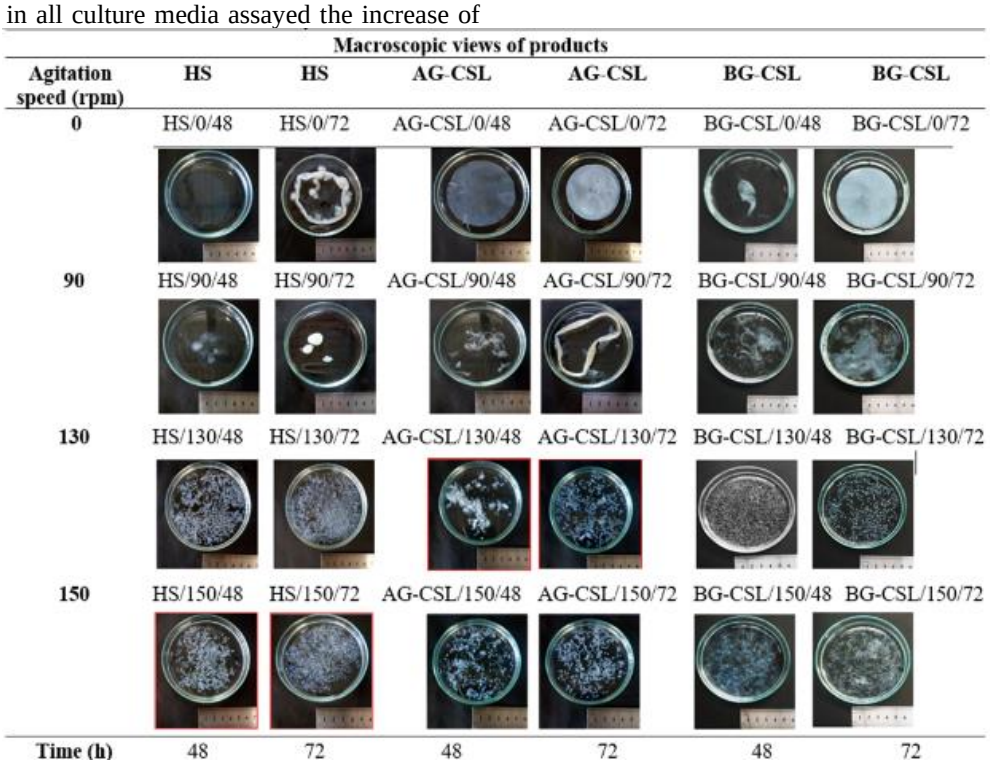

Figure 2. Representative macroscopic views of BNC products obtained at varying culture media, production time and fermentation mode.

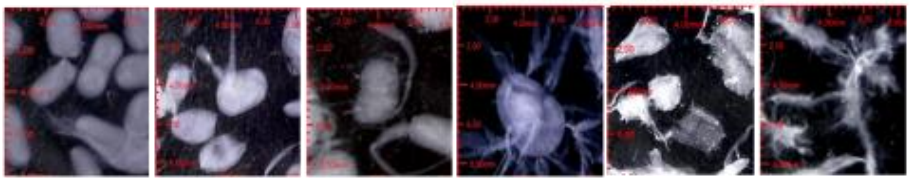

Figure 3. Representative pellet morphologies obtained after 72 h in varying culture media using 130 and 150 rpm.

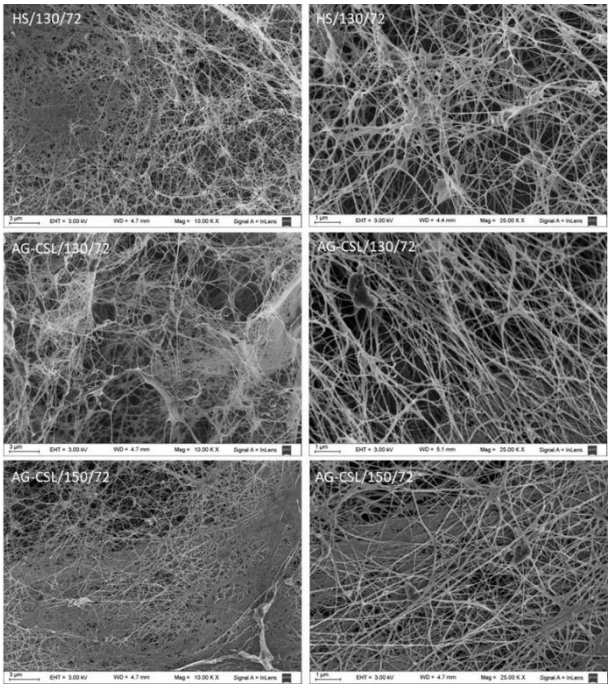

Figure 4. FESEM images of BNC samples produced at 72 h in HS and AG-CSL media.

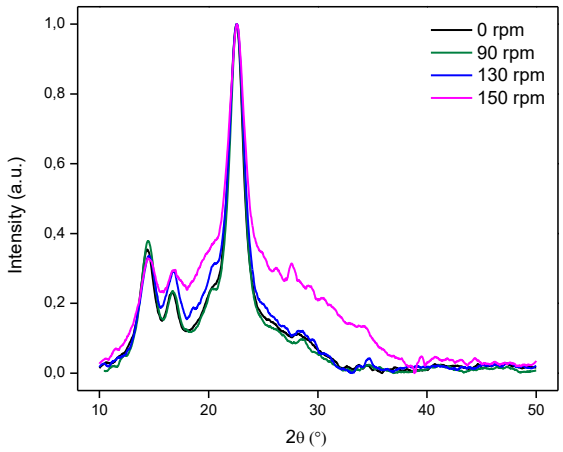

Figure 5. X-ray diffraction data from BNC samples produced at 72 h in AG-CSL media with varying stirring speed.

Table 2. Crystallinity index (CI) determined by use of Segal's method for BNC samples produced at 72h in AG-CSL media. Effect of stirring speed.

BNC sample	CI (%)
AG-CSL/0/72	90
AG-CSL/90/72	89
AG-CSL/130/72	86
AG-CSL/150/72	78

intense peaks corresponding to the (021) lattice plane centered at 2θ = 20.3° and (040) plane centered at 2θ = 34.7°, both also characteristic of Cellulose I, could be seen in some of the samples.

The diffractograms of BNC produced in static culture, at 90 rpm and 130 rpm were quite similar, suggesting that these stirring speeds did not result in detectable changes in the packing of cellulose nanoribbons. On the other hand, at the highest stirring speed used (*i.e.* 150 rpm), the X-ray diffractogram illustrates a significant change with respect to that of BNC obtained in static conditions, suggesting that the stresses generated at such agitation speed might have affected the packing of cellulose chains, leading to a less crystalline pattern.

Table 2 further confirms the observations described in terms of the crystallinity index values (CI) determined by use of Segal's equation, with a CI value for BNC pellets obtained at 150 rpm significantly smaller than the ones measured for BNC produced in static culture and also for BNC pellets produced at lower stirring levels. The observations made are in line with previous contributions which have illustrated that using a definite strain, fermentation time and medium composition, the crystallinity index value of BNC produced in static condition was higher than that obtained in agitated culture (Aydın and Aksoy, 2014; Sarkono *et al.*, 2014, Singhsa *et al.*, 2018). Results have been often associated with agitation interfering strongly in nascent microfibrils crystallization and hydrogen bonding network formation (Czaja *et al.*, 2004; Sarkono *et al.*, 2014; Singhsa *et al.*, 2018).

D. BNC specific surface area

The specific surface area (SSA) and the porosity of BNC depend on the arrangement of the cellulose nanoribbons within the pellicles or pellets produced in either static or dynamic culture. Closely arranged fibrils lead to low porosity and reduced total surface area (Gao *et al.*, 2011; Guo and Catchmark, 2012; Ul-Islam *et al.*, 2012), whereas more loosely packed samples often result in increased specific surface area values. SSA determination is very relevant for several BNC applications for which compounds adsorption and release, water holding capacity, and/or cell attachment and growth, are key aspects (Gao *et al.*, 2011; Ul-Islam *et al.*, 2012). In this context, the SSA of BNC has been previously measured by Congo red adsorption (Corzo Salinas *et al.*, 2021) and, more frequently, by nitrogen gas sorption analysis with SSA calculation according to the multipoint Brunauer-Emmett-Teller (BET) method (Ul-Islam *et al.*, 2012; Yin *et al.*, 2012). It is worth noting that absolute results from both techniques are not expected to be similar since hornification (*i.e.* the irreversible formation of hydrogen bonds between fibrils during samples dehydration) is known to

severely condition the extent of fibrils aggregation and thus the SSA values determined (Corzo Salinas *et al*., 2021).

Table 3 illustrates the effect of stirring speed on the SSA of BNC assemblies obtained in AG-CSL medium at 72h. Results evidence that BNC samples produced in static culture led to the lowest SSA value, which is actually within the order of the SSA values reported for freeze-dried BNC membranes by other authors (Cacicedo *et al*., 2016; Yin *et al*., 2012).

Table 3. Specific surface area (SSA) determined by BET method for freeze-dried BNC samples produced at 72h in AG-CSL media. Effect of stirring speed.

BNC sample	SSA (m^2/g)
AG-CSL/0/72	32±0,2
AG-CSL/90/72	72±2,7
AG-CSL/130/72	225±7,7
AG-CSL/150/72	192±19,8

Besides the described differences in BNC products size and resulting total external area, results indicate that in static culture pellicles are formed by a more compact fibrils network. On the other hand, agitation induced a significant increase in the SSA values of BNC, specially at high stirring rate, suggesting that agitation modified the fibrillar structure of the BNC network formed, inducing more loosely packed structures. Quantitative SSA results are in agreement with previous contributions in which the generation of looser nanoribbons network structures upon stirring had been qualitatively illustrated by SEM analysis (Aydın and Aksoy, 2014; Sarkono *et al*., 2014; Singhsa *et al*., 2018).

IV. CONCLUSIONS

In the current contribution BNC pellicles and BNC pellets were produced in the standard HS medium as well as in alternative less costly glycerol-corn steep liquor culture media. Results showed that the alternative media used led to significantly higher BNC production, which has interesting economic implications for potential scale-up, especially when the use of glycerol remaining from increasing biodiesel production is considered. On the other hand, results evidenced that the stirring speed used significantly influenced both the amount and the characteristics of the BNC produced, *e.g.* shape, size, crystallinity and cellulose nanoribbons network packing. Modulation of BNC pellets size, crystallinity and specific surface area by proper tailoring of the stirring rate used during dynamic fermentation, is an interesting strategy for growing applications of discrete particles of BNC such as adsorbent products (heavy metal, dyes), scaffolds (cell attachment and growth), drug carriers, and catalyst/biocatalyst support materials.

NOMENCLATURE

AG: analytical glycerol
AG-CSL: analytical glycerol/corn steep liquor fermentation medium
BG: glycerol remaining from biodiesel production
BG-CSL: glycerol remaining from biodiesel production/corn steep liquor fermentation medium
BNC: bacterial nanocellulose
CI: crystallinity index
CSL: corn steep liquor
FESEM: field emission scanning electron microscopy
HS: standard Hestrin and Schramm fermentation medium
SSA: specific surface area
XRD: X-ray diffraction analysis

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