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# **In situ production of bacterial cellulose to economically improve recycled paper properties**

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## **ABSTRACT**

This study focusses on the in-situ production of bacterial cellulose in recycled pulps to increase the quality of fibers in the suspension. The effect of different dosages of the upgraded pulp on the mechanical, physical and optical properties of handsheets was assessed. Papers produced with pulps cultivated in agitation exhibited increments in both tensile and tear indexes of 12.2% and 14.2%, respectively. Thus, flexibility of the paper was also improved. On the other hand, pulps enhanced with static culture fail to improve tensile index of paper, while tear index was increased by 12.4%. The production mechanism for both types of culture was proposed. In agitated culture, bacteria were found to coat the primary fibers, improving their quality. In the case of static culture, heterogeneous systems were observed since recycled fibers tended to sediment while bacteria moved to the surface of the culture broth in search of oxygen. Hence, the in situ production of BC with recycled fibers can, therefore, be an alternative to replace conventional paper strengthening agents. The results attained indicate that the in-situ production of upgraded pulps can be implemented in paper mills cultivating pulp streams sterilized through low cost, non-exhaustive operations, such as ozone or ultraviolet radiation.

25    *Keywords: bacterial cellulose, recycled paper, strength, in situ culture, mechanical*  
26    *properties, industrial application*

## 1. INTRODUCTION

Recycled paper (RP) global demand has increased in recent years, worsening the quality of the recovered paper [1]. As consequence of the fiber hornification process in each recycling cycle, the paper mechanical properties are negatively affected [2, 3]. A lower inter-fiber and filler-fiber bonding capacity, and a reduction in the ability to swell when fibers are re-suspended in water are produced, which reduce the paper strength [4]. Recently, paper strengthening agents based on nanocelluloses have been increasingly used, even though their production is still expensive and nanocellulose fiber dispersion is still an issue [3].

Bacterial cellulose (BC) presents several advantages compared to plant cellulose, such as high purity, high polymerization degree and high crystallinity [5, 6]. These exceptional properties make BC a great candidate to be used as strengthening agent of several materials [7]. It can be obtained by aerobic bacteria of the genus *Komagataeibacter*, mainly isolated from rotten fruits and wastes of the vinegar fermentation [8]. Between all culture methods, static and agitated modes have been the most used due to their simplicity and high yield [9]. In static cultures, bacteria tend to move towards the surface of the culture broth. Thus, BC is synthesized on the surface of shallow trays as a pellicle with higher density on the side exposed to air [10]. In view of this behavior, a large space is required to reach a worthwhile amount, fact that usually makes this type of process difficult to scale-up [5]. With the implementation of agitation, the mass transfer rate is improved, increasing the oxygen availability within the whole culture broth [11]. However, some authors have published different studies about the appearance of a genetic mutation of bacteria of the described genus, due to the high shear stress provoked by high agitation and aeration [12, 13]. According to these authors, this spontaneous mutation deactivates essential enzymes involved in the cellulose synthesis, reducing the cellulose

production rate [14]. In this sense, bacteria synthesize other water-soluble polysaccharides with lower strength such as acetan, levan or xylan, which are composed of the same starter molecule than BC [15].

Between the different strategies used to obtain composites made of BC, the in-situ culture of bacteria with other components is found the most useful [16]. This fact could trigger a reduction in the required time to produce a nanocomposite, as well as a better and more compact conformability [17]. In this way, mechanical and barrier properties, as well as water holding capacity of the materials added to the culture broth have been improved. BC has been in-situ produced with potato starch [18], poly-3-hydroxybutyrate [16], bentonite [19], graphene oxide [20, 21], glyoxal [22], carboxymethyl cellulose [23, 24], gelatin [25] and carbon nanotubes [26].

Materials strengthening by BC in-situ production presents several advantages compared to the addition of cellulose nanofibers (CNF) and cellulose nanocrystals (CNC) to the same materials, which can be grouped in the minimization of costs [10]. Nutrients cost can be reduced to almost zero by using industrial residues or wastes containing sugars, such as konjac powder hydrolyzate [27] or waste from beer fermentation broth [13], which also contributes to the circular economy aims.

However, this approach has been seldom published in the papermaking field, and deeper studies are necessary before considering its industrial implementation [28, 29]. In this work, the in-situ production of BC with recycled fibers to improve the mechanical properties of RP has been studied. First, the effect of the culture mode, in terms of static or agitated methodologies, on the mechanical, physical and optical properties has been assessed. Moreover, a production mechanism for both methods has been proposed. Finally, different culture volumes and times have been studied to assess the influence of the oxygen availability. These results provide new insights for the papermaking

application on the culture modes, as well as on the main variables affecting the BC productivity (culture and time volume).

## **2. MATERIALS AND METHODS**

### ***2.1 Materials***

The bacterial strain *Komagataeibacter sucrofermentans* CECT 7291, used in this study, was obtained from the Spanish Type Culture Collection (CECT). To retain the fibers, a three-component retention system (C-F-B), commonly used in paper mills, was selected. Polyamine (C) with a high molecular weight and a cationic charge density of 0.035 meq/g was used as coagulant; polyacrylamide (F) with a high molecular weight and a cationic charge density of 3.66 meq/g was used as flocculant; and hydrated bentonite clay (B) was used as microparticle. These products were supplied by BASF (Ludwigshafen, Germany). Recycled newsprint (NP) and magazine papers (MG) were kindly supplied by Holmen Paper (Madrid, Spain). Nutrients, such as fructose, yeast extract and peptone, as well as other reactants like NaCl, KCl,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{NaHCO}_3$  and NaOH were of analytical grade and supplied by Sigma-Aldrich.

### ***2.2 Bacterial growth and culture media preparation***

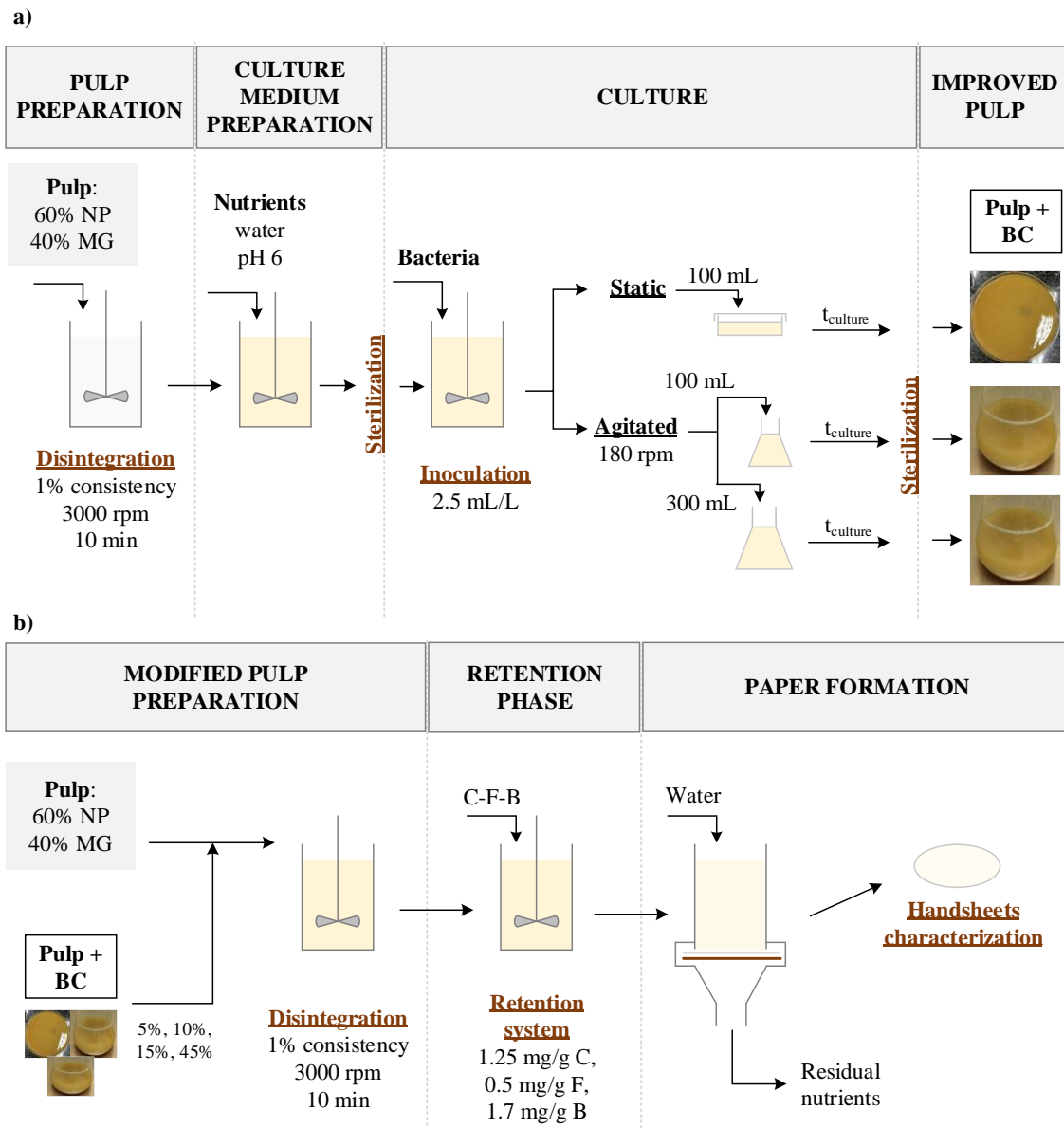
Cell growth and bacteria isolation were developed according to the procedure previously described [30]. The bacterial suspension was prepared with Ringer's solution by adjusting optical density to 0.59–0.64 at the wavelength of 600 nm.

A pulp suspension of 0.5% consistency was prepared by pulping 60% NP and 40% MG papers using a Messmer pulp disintegrator (Mavis Engineering Ltd, London, UK) at 3000 rpm for 10 min [3]. Then, 20 g/L fructose, 5 g/L yeast extract and 3 g/L peptone were added to the pulp suspension and pH was adjusted to 6 by the addition of diluted HCl.

The prepared suspension was autoclaved at 121 °C. Once it reached room temperature, 2.5 mL of the bacterial suspension were added to inoculate each liter of pulp.

### ***2.3 In-situ culture***

For static culture, 100 mL of the inoculated suspension were poured into 100 mL Petri dishes and let to culture at 30 °C. For agitated culture, either 100 mL of the culture suspension were added to 250 mL Erlenmeyer flasks or 300 mL to 500 mL Erlenmeyer flasks, being placed into a Certomat IS orbital incubator manufactured by Sartorius Stedim Biotech GmbH (Goettingen, Germany) at 180 rpm and 30 °C. Cultures were carried out for 6, 12, 24, 36 and 48h, autoclaving the samples at each time before using it to prepare handsheets. Figure 1a shows a simplified scheme for an easier understanding of the process.



**Figure 1.** a) Procedure developed to culture bacteria in presence of fibers, and b) Procedure carried out to form handsheets at different proportions of the improved fibers composed of pulp and BC.

## 2.4 Handsheets formation

To assess the effect of the culture mode on the mechanical, physical and optical properties of paper, different culture flasks at each time were autocleaved to remove bacteria and mixed with the corresponding amount of pulp to reach a total of 1 L at 1% of consistency. Developed experiments and nomenclature used for each case are shown in Table 1. Then,

the mixture was pulped at 3000 rpm for 10 minutes. The retention system, (1.25 mg/g of C, 0.5 mg/g of F and 1.7 mg/g of B) was added previously to handsheet formation. Then, handsheets with a basis weight of 60 g/m<sup>2</sup> (ISO 5269/2 (2004)) were prepared with a normalized Rapid-Köthen handsheet former (PTI, Vorchdorf, Austria). A schematic view of the procedure is shown in Figure 1b.

**Table 1.** Used nomenclature for each experiment, corresponding to different amounts of improved pulp, in a defined number of parallel culture flasks at different volumes

Nomenclature	Improved pulp	Number of parallel culture flasks	Culture volume of each flask
5% (1)	5%	1	100 mL
10% (2)	10%	2	100 mL
15% (3)	15%	3	100 mL
15% (1)	15%	1	300 mL
45% (3)	45%	3	300 mL

## 2.5 Handsheet characterization

Handsheet grammage was determined according to ISO 536. Formation homogeneity was evaluated by the standard deviation of 400 microgrammage measurements by using a Beta formation tester (Ambertec, Espoo Finland). Mechanical, physical and optical properties, such as tensile and tear strengths, strain at break, porosity, thickness, ISO Brightness, and CIE L\*, a\* and b\* were measured with an AUTOLINE 300 from Lorentzen & Wettre (Stockholm, Sweden). Tensile (TI) and tear indexes were determined as the ratio between the corresponding strength and its grammage.

Morphology of the handsheets was analyzed by scanning electron microscopy (SEM), with a JEOL JSM 6335F at an accelerating voltage of 15 kV. These analyses were carried out at the *National Center of Electronic Microscopy of Spain*.

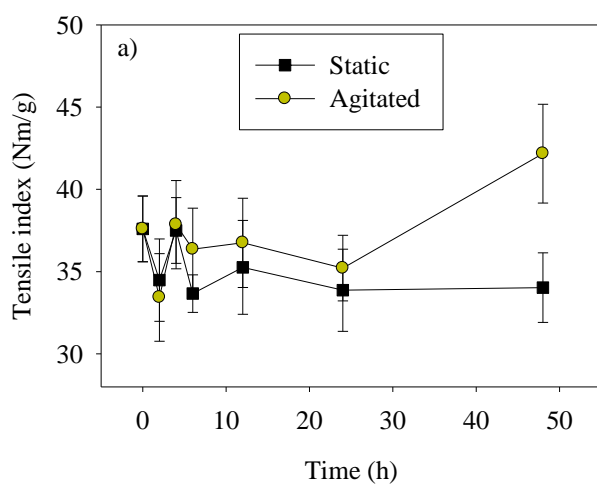
### 3. RESULTS AND DISCUSSION

#### *3.1 Effect of culture mode on paper properties*

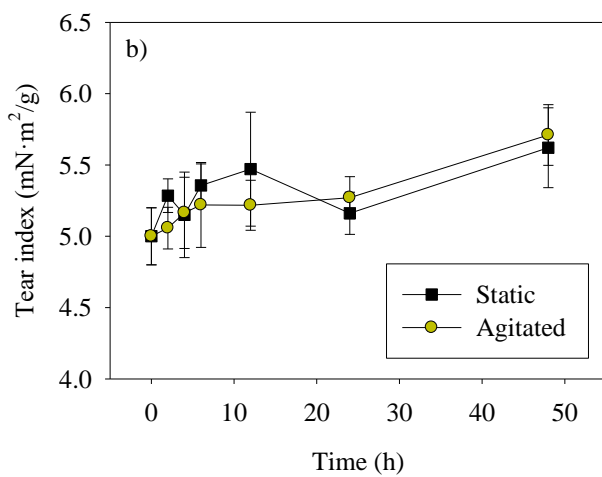
##### *3.1.1 Mechanical properties*

Handsheets prepared at the dosage of 10% (2) by both static and agitated cultures were used in order to compare the effect of the culture mode on the paper properties. Evolution of the handsheets TI with the culture time is shown in Figure 2a. Before 24 h of culture, handsheets prepared with improved fibers cultured in both static and agitated modes, presented a similar behavior. The control experiment (handsheets with the same amount of the cultured suspension but without being inoculated) was 37.6 Nm/g, represented in Figure 2a as zero time. Results are in agreement with data from Jung, et al. [31], who established that cell growth gets a maximum after 24 h of culture, starting to increase the BC productivity in that moment.

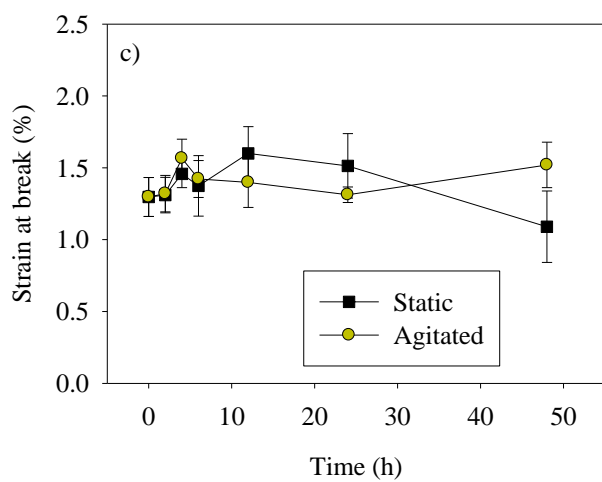
At 48h of incubation, TI of handsheets increased up to 42.2 Nm/g for agitated culture, meaning an increase of 12.2%. At this time, the number of cell colonies keeps constant, thus increasing the BC productivity. However, this is not as positive for static cultures, where TI remained below the control value (34.1 Nm/g). The lower movement of bacteria in static cultures limit the nutrients availability, thus decreasing the cell growth rate and therefore the BC productivity. However, it is highly likely that increasing the culture time, handsheets TI will be enhanced since the BC produced by static cultures usually presents better properties in terms of crystallinity and fibers length.



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**Figure 2.** Effect of culture time on the mechanical properties of paper in both static and agitated mode: a) Tensile index, b) Tear index and c) Strain at break.

It is well known that tensile and tear strengths are compromised when CNF are added as strengthening agents, since when one is improved the other is negatively affected [32]. This fact has been assigned to the high number of hydrogen bonds between CNF and paper fibers [33]. However, as it was observed in a previous study [30], when nanofibrillation was not so high, not only hydrogen bonding drives the retention of nanocellulose, but also physical retention in the fiber gaps. In this case, the BCNF clusters added to the pulp not only reinforced the paper, but also made the paper more flexible by favoring the transmission of the breaking force throughout the paper and, consequently, improving the tear index.

In this study, tear index of handsheets was improved with the culture time in both agitated and static systems (Figure 2b). Comparing with Figure 2a, tear index variation results inversely related to TI when culture is carried out in static mode. However, in the case of agitated culture, TI remained almost invariable when time was below 24 h, while tear index kept increasing until 48 h (reaching a maximum increment of 14.2% at 48h).

As it was explained before, BC produced in static mode formed a web on the surface of the culture broth [8]. On the other side, primary cellulose fibers were likely to sediment. Then, the bonding between BC and primary fibers could have taken place during the pulping after mixing with the pulp, but seldom during the culture.

On the opposite side, it seems that in agitated culture, bacteria had preference to grow on the surface of the cellulose fibers, probably having a higher oxygen availability. In this way, BC could have been synthesized as coating of these fibers, not only by hydrogen

bonding but also as a physical covering. This fact triggers a higher handsheets flexibility, since the inter-fiber force transmission is developed in a larger area [33].

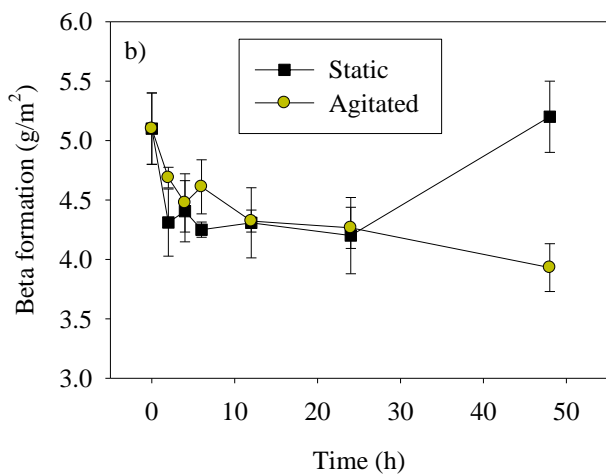
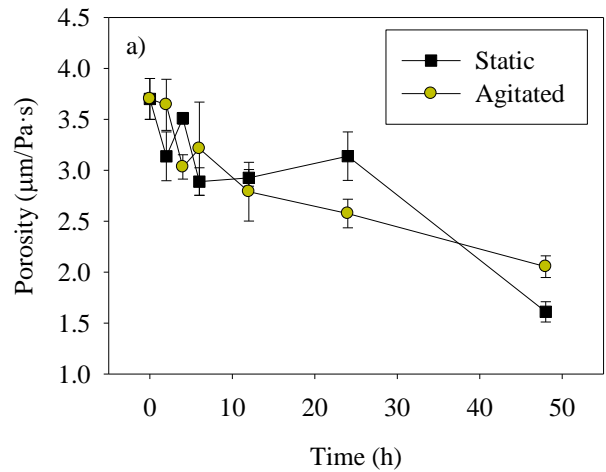
Strain at break results are represented in Figure 2c. When BC was produced in static culture, strain at break increased by 24.1% the first 12 h, but it was reduced by 15.5% after 48 h of culture compared to the initial value. This fact could be related to the irregular formation of the handsheets, since the synthetized BC pellicles were difficult to disperse [30]. However, in agitated culture, strain at break increased by 8.5%, when time was below 24h, and by 17.8% at 48h, with also agrees with the higher flexibility found based on TI and tear index results.

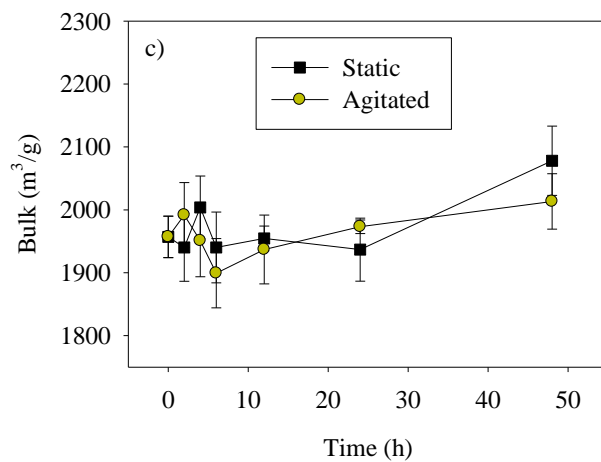
Other important issue that can affect the mechanical performance of paper is the fact that the BC produced by the two cell culture methods are different [8]. Static cultures provide a higher BC crystallinity and length since bacteria are free to move through the surface of the culture broth. These two factors would induce a significant enhancement in paper strength if the BC nanofibers could be completely disperse. However, this is one of the main drawbacks of culturing bacteria in static mode, due to the high entanglement of nanofibers in the BC pellicle [30]. On the other hand, more amorphous and short chains of BC are produced in agitated systems, due to the shearing of the culture broth that can break BC nanofibers while they are being formed. In view of these properties, a worse improvement would be expected for agitated cultures compared to static. However, a higher nanofiber dispersion could be reached, thus allowing a greater effect of BC on the paper mechanical performance.

### *3.1.2 Physical properties*

Nanocelluloses are known to decrease the porosity of handsheets since they are mainly fitted within the gaps between fibers, forming a more compact structure, thus improving

barrier properties [34]. As shown in Figure 3a, porosity of handsheets formed by culturing  
 the pulp in static mode presented a decreasing tendency with culture time. However,  
 porosity decreased in a smoother way in the case of agitated cultures, reaching a  
 decrement of 44.6% when culture time was 48 h.





**Figure 3.** Effect of culture time on the physical properties of paper in both static and agitated mode: a) Porosity, b) Beta formation and c) Bulk.

Beta formation measurement shows the homogeneity of the handsheets as the standard deviation of the microgrammage measured in different points of the sample. According to Figure 3b, formation of handsheets prepared with the static cultured improved pulp was more homogeneous when time was below 24 h, since beta formation decreased by 15.7%. However, the formation of a pellicle of BCNF during culture at high culture time (48 h) triggered more irregular papers, increasing the beta formation index by 2% compared to the initial sample.

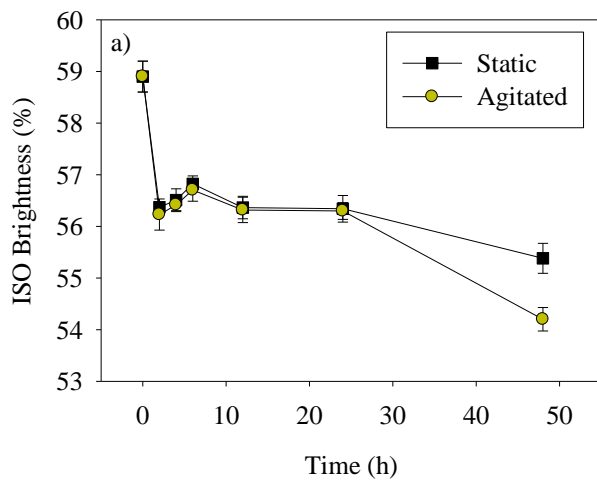
In the case of agitated cultures, beta formation presented a smoother decreasing tendency, meaning that the production of BC was carried out homogeneously within the pulp. Even at low culture times, below 6 h, this parameter was reduced by 12.2%, and by 20% at 48 h. These results for agitated cultures present a high advantage respect to those of static mode, since the control of the handsheet properties can be carried out in a more confidence way.

Figure 3c shows the evolution of the handsheets bulk with the culture time. The bulk is defined as the ratio between the thickness and the grammage. In this case, a similar

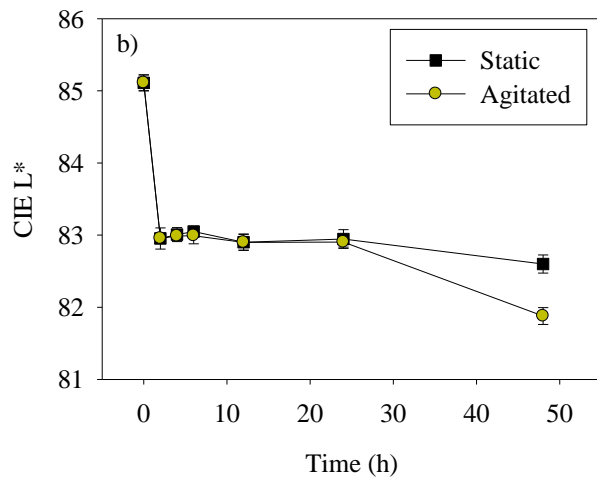
behavior was observed between both static and agitated cultures, decreasing the value of this parameter at short times followed by an increase after 12 h. The first reduction was assigned to the replacement of the original fibers by improved fibers of either BC alone (static mode) or fibers covered by BC (agitated mode). It resulted in a reduction of the handsheet thickness while keeping the grammage without variation [35]. Then, when culture time was 48 h, the bulk further increased by 6.2% and 2.8% for static and agitated cultures, respectively. Depending on the final use of the paper, a low bulk could be inconvenient, since it entails a low paper manageability [1]. Therefore, a further optimization should be carried out in the case of newsprint, magazine or office papers.

### *3.1.3 Optical properties*

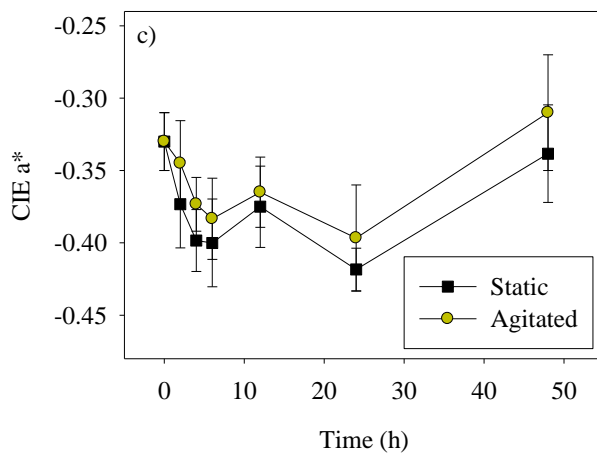
Optical properties were also affected by the addition of improved fibers to the pulp, reducing the ISO Brightness of handsheets when the pulp was cultured even for short times (Figure 4a). This effect could be assigned to the presence of nutrients in the handsheets, some of them of brown color (yeast extract and peptone). Nutrients are supposed to pass through the filtering mesh when handsheets are being formed, due to the small particle size. However, as BC fibers diameter is in the nano-scale, some nutrient particles can remain as impurities within the paper, thus causing a detrimental effect on optical properties. This fact was much more intense at high culture time (48 h), since it was reduced by 3.5 and 4.7 points for static and agitated cultures, respectively. As the produced BC amount through static mode is lower than in agitated mode, the nutrients present in these handsheets are scarcer and thus ISO Brightness is not as affected.



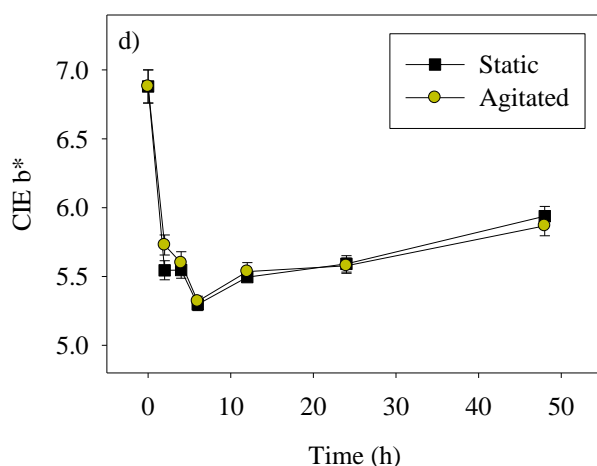
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**Figure 4.** Effect of culture time on the optical properties of paper in both static and agitated mode: a) ISO Brightness, b) CIE L\*, c) CIA a\* and d) CIE b\*.

The colorimetric constants showed a narrow trend between the values for static and agitated cultures. In this way, CIE L\* and CIE b\* decreased by 2.2 and 1.5 points, respectively, after 2 h of culture (Figure 4b and d). It means that handsheets were tending to black and blue colors with the longer culture time. Respect to CIE a\* (Figure 4c), it was not very affected by the nutrients present within the paper, varying slightly in the range of -0.32 to -0.4. The reason for this decrement in CIE a\* could be mainly due to the darkness of the culture media. Nevertheless, as it can be observed, the darkness of the handsheets is not as important as the color change.

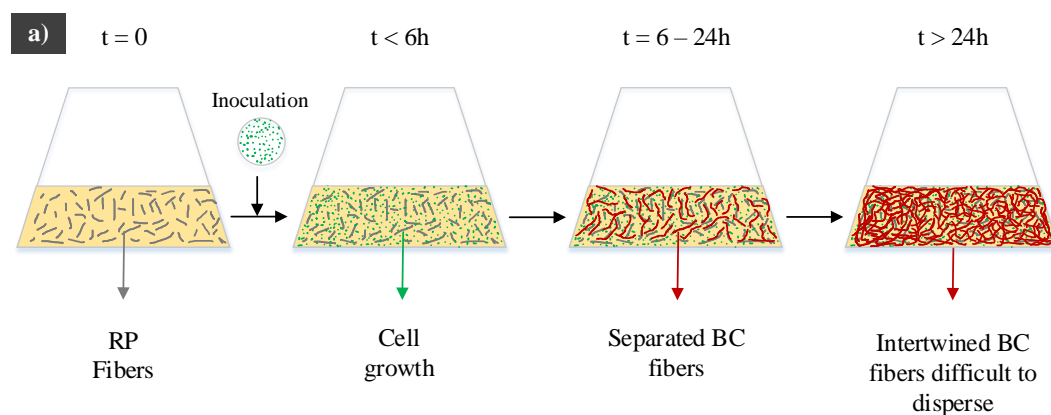
### 3.2 Static and agitated culture: production mechanism

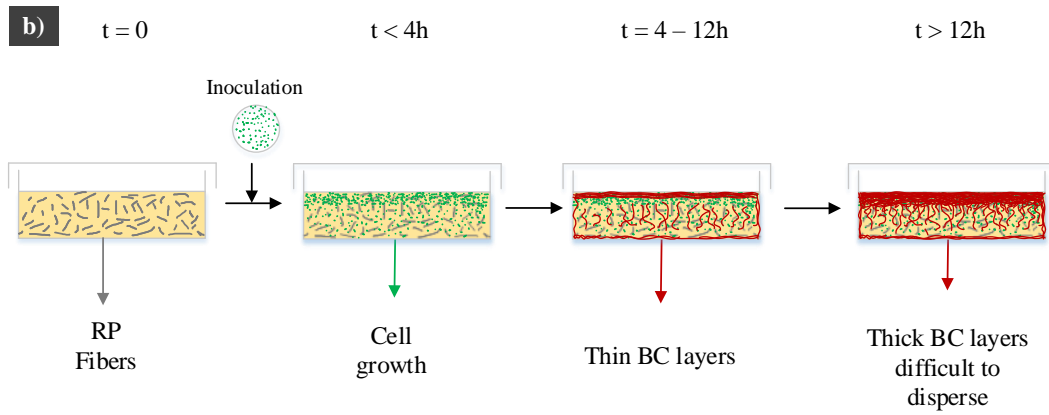
A schematic illustration of BC synthesis in the presence of fibers for both agitated and static cultures is shown in Figure 5.

In the case of agitated culture (Figure 5a), RP fibers are distributed homogeneously through the culture broth at zero time, as observed in Figure 6a, where a macroscopic view of the culture broth is displayed. After the pulp was inoculated, mainly cell growth

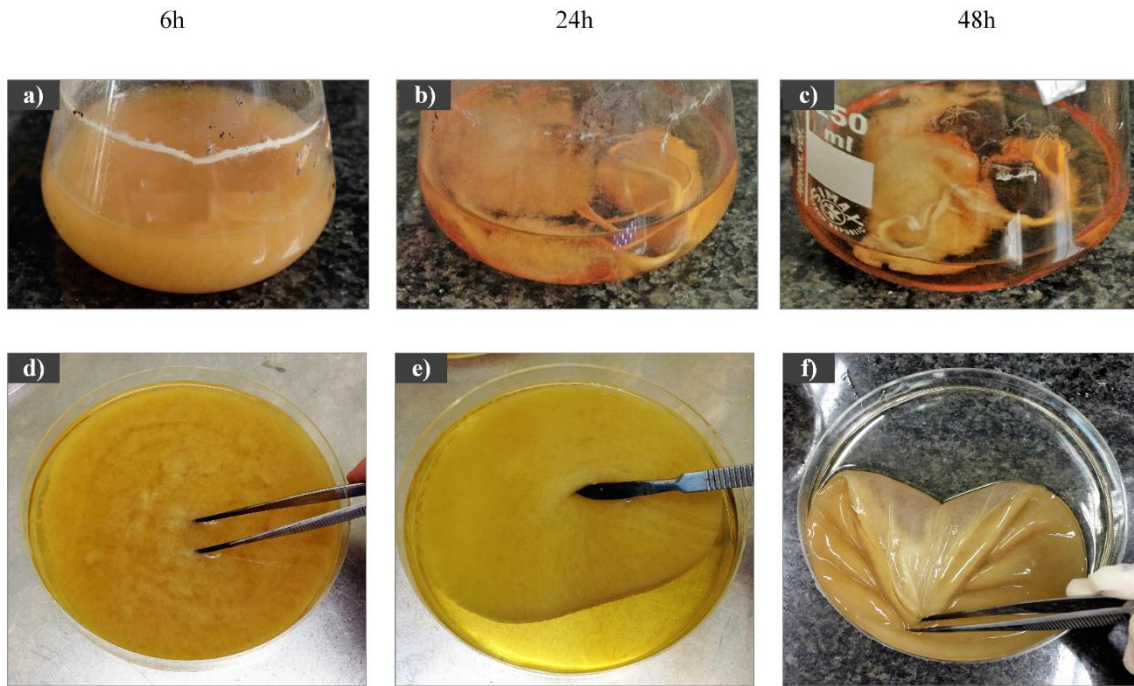
took place for the first 6 h of culture. However, when the culture time was over 6 h, the BC fibers started to make appearance (Figure 6b). Considering the fact that aerobic bacteria have the preference for zones with high oxygen availability, it is highly probable that they tend to grow on the fibers surface. Thus, BC would be likely synthesized as a coating of these fibers, fixing the flaws of the recycled fibers. With the orbital agitation of the culture, bacteria keep moving through the broth while they are producing BC, so finally a network composed of coated fibers was created. When this network was further disintegrated, the improved fibers could be separated. Then, mechanical properties of handsheets are highly probable to be improved.

Moreover, when culture time was over 24 h, those networks are formed with a more compact and strong structure, being usually difficult to separate (Figure 6c). Therefore, big clusters are the result of the pulp disintegration, with heterogeneous shape and composition. Then, irregular handsheets with areas of a high amount of BC and others with lack of fibers or even holes are obtained. As a conclusion, it can be confirmed that culture time is a key parameter for the in-situ production of BC with recycled fibers by agitated culture, and has to be optimized to reach a worth improvement in paper mechanical properties.





**Figure 5.** Proposed mechanism for in-situ culture of bacteria with cellulosic fibers in a) agitated mode and b) static mode.

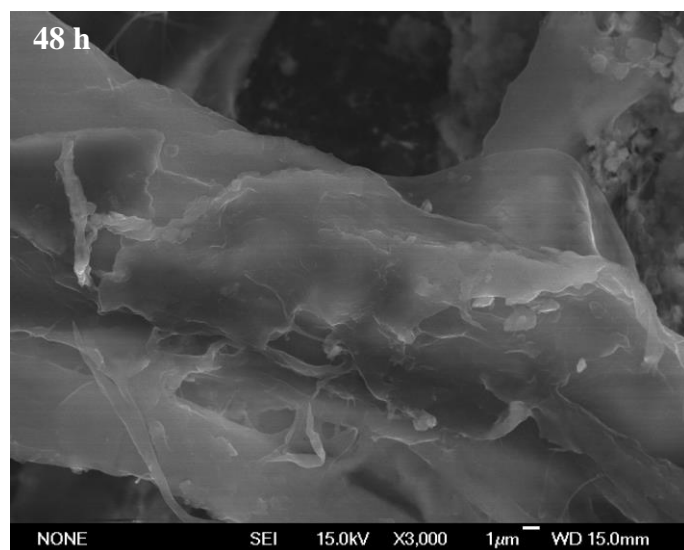
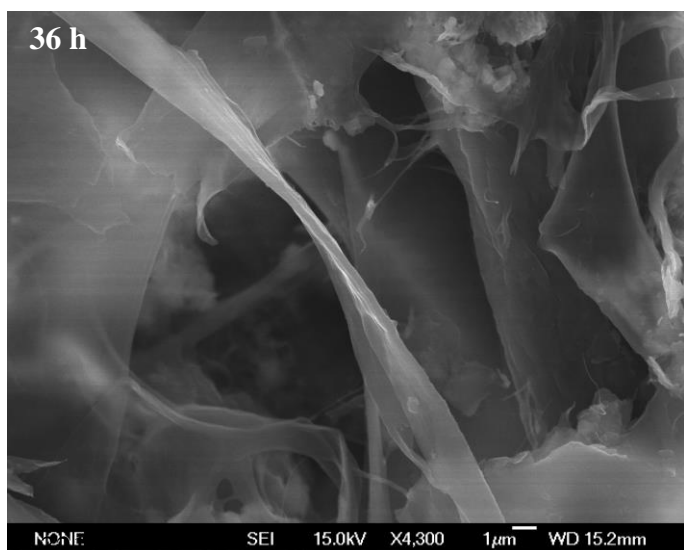
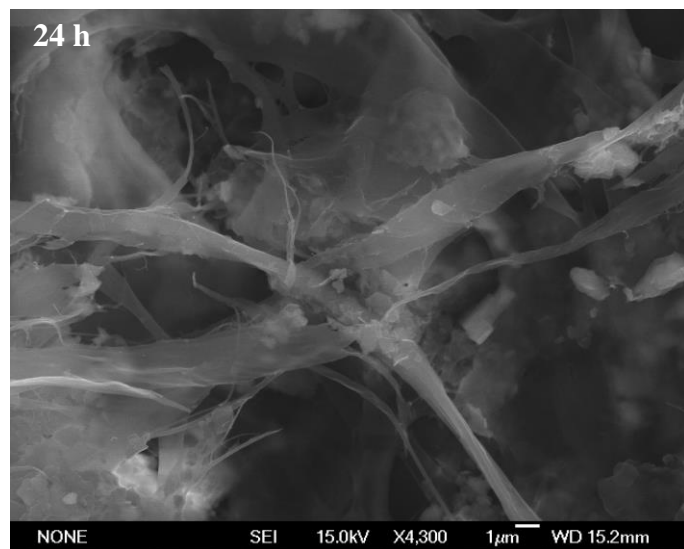
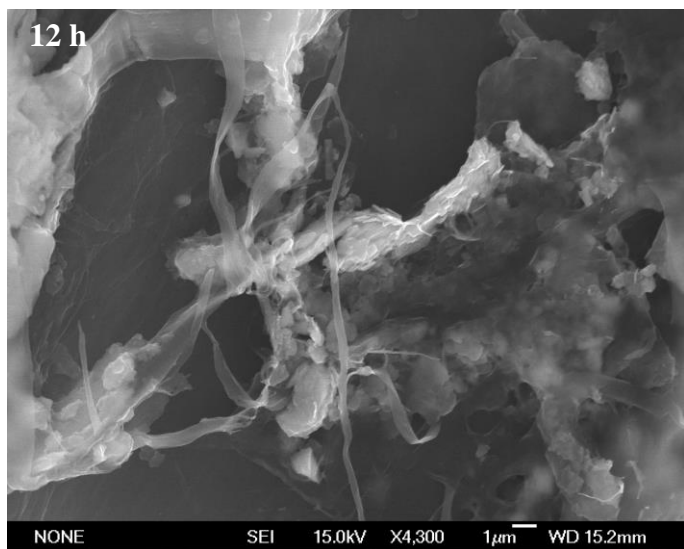
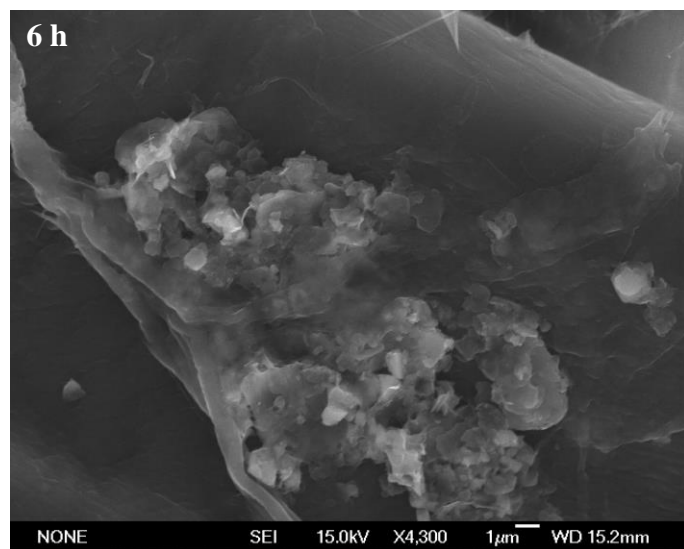
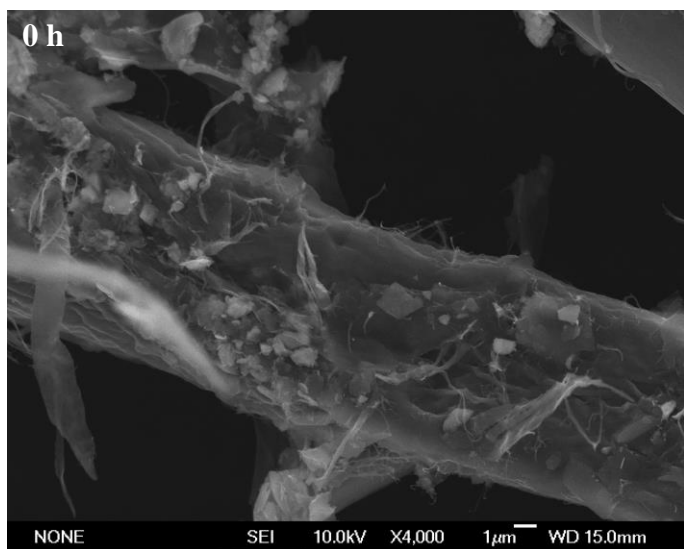


**Figure 6.** Images of agitated (a, b and c) and static (d, e and f) in-situ cultures after 6 (a and d), 24 (b and e) and 48 h (c and f) of culture time.

In the case of static culture, while recycled fibers tended to sediment, bacteria moved to the surface of the culture broth in search of oxygen (Figure 5b). In this case, as published previously [11], cell growth is slower, but as most of bacteria are concentrated on the surface of the liquid phase, BC production can be detected even before 4 h of culture. At

301 this time, a BC thin gelatinous pellicle is observed, as shown in Figure 6d. When the  
302 culture time increased to more than 12 h, a thicker BC pellicle with a denser surface on  
303 the side exposed to air is formed [10]. Figure 6e showed as the BC pellicle adsorbed  
304 almost all the fibers added to the culture, deducing that not only BC was being produced  
305 on the surface of the culture broth, but also on the surface of the Petri dish (although in a  
306 lower extent). This fact was observed in Figure 6f, when after 48h of culture, a thick and  
307 gelatinous membrane was produced at the top of the culture and a thinner but resistant  
308 membrane was also formed around the culture broth, wrapping all the fibers and medium  
309 inside (Figure 6f).

310 SEM images of the handsheets formed by the combination of agitated cultured pulp at the  
311 dosage of 10% (2) and recycled fibers are shown in Figure 7. When handsheets were  
312 formed before the inoculation of the pulp (shown in graphs as 0 h), individual primary  
313 fibers were observed with some mineral fillers attached to their surface. Also, it is worth  
314 mentioning that these fibers show a rough surface, mainly due to the deterioration caused  
315 by the recycling cycles. As explained before, when bacteria were cultured with the pulp  
316 for 6 h, some BC fibers were produced as a coating of these fibers, easing the attachment  
317 of the mineral fillers within the paper sheet. At higher culture time, some bridges of BC  
318 were detected between recycled fibers. Finally, it is observed in Figure 7 at 48 h that not  
319 only the fibers were covered, but also they were joint by BC, which is shown in the  
320 micrographs as a smooth and homogeneous surface.



**Figure 7.** SEM micrographs of BC/Recycled fibers handsheets produced by in-situ culture of bacteria in agitated mode.

Taking all these aspects into account, it can be concluded that static culture of bacteria in-situ with fibers was faster than agitated culture, but heterogeneous systems were produced. In addition, the formed BC pellicles were difficult to disintegrate, which caused a bad handsheet formation, as well as a mechanical properties worsening. On the other side, agitated cultures resulted in efficient systems, since BC was found to cover the primary recycled fibers, compensating for the fiber damage. Moreover, the enhanced paper flexibility observed previously when pure BC was added as a strength additive to the pulp [30] was also found in this culture mode. Therefore, a deeper study of the effect of the culture volume on mechanical, physical and optical properties of the RP was carried out in agitated mode.

### ***3.3 Effect of the culture volume on paper properties***

Nutrients were supposed to pass through the filter when handsheets were being formed, due to their small size, together with the fact that they were dissolved. However, the residual amount present in handsheets was observed to affect paper properties, so they were taken into account for all explanations.

Zero time values showed in Figure 8 were those obtained with the addition of cultured pulp without inoculation at the different dosages. They show the tendency of TI to improve with a higher dosage of the cultured pulp. Thus, for the dosages of 5%, 15% and 45%, TI increased by 8.5%, 15.3% and 28.4% compared to handsheets made of pulp without nutrients. Fructose, the carbon source used in this study, is surrounded by hydroxyl groups, which makes possible the bond between them and the cellulose chains

by hydrogen bonding [36]. In this way, the size of gaps among the cellulose fibers are reduced, thus increasing the tensile strength of the material.

When the cultured pulp dosage was 5% (1), the handsheets TI was found to increase with the culture time for the first hours, reaching improvements of 6.8% and 10.8% at 6 and 12 h. After that, TI decreased due to the heterogeneous formation of the handsheets, which concentrated the improved fibers with BC in a specific area of the handsheets. This behavior was related to the BC production mechanism in agitated culture previously explained, since the improvement in mechanical properties was decreased after 24 h.

With the addition of the triple amount of the cultured pulp, 15% (3), TI reached 39.0 Nm/g, which is the same value as the maximum TI for 5% (1), but 6 h before. However, the increment respect to the value at zero time was only 3.4%, meaning that the main parameter affecting the enhancement in the mechanical properties was the amount of nutrients present within the handsheets. Thus, although the dosage was triplicate, TI was not as improved as expected.

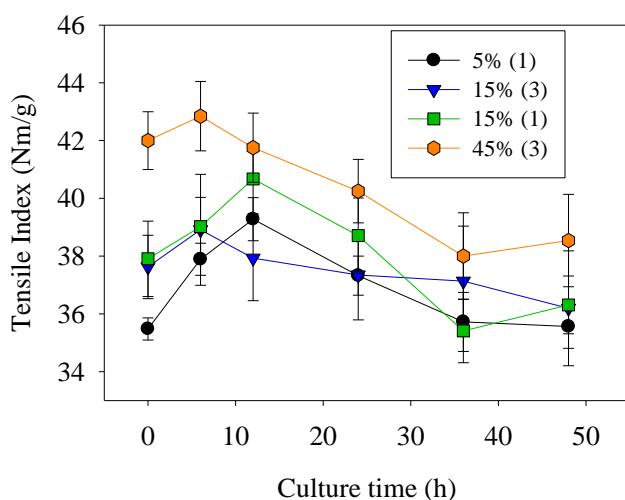
Nevertheless, when the same volume was cultured in one single flask (15% (1)), the results were more satisfactory. The highest TI value was obtained at 12 h (40.7 Nm/g), reaching an increment of 7.4% compared to the TI for zero time at this dosage. Then, after 24 h of culture, the TI increment was strongly reduced to 2.1% and even reaching a decrement after that. As mentioned before, the formation of a high amount of BC difficult to disperse together with a lower amount of nutrients could be the causes of this worsening.

Compared to 15% (3), a higher enhancement in the mechanical performance was achieved for culture times below 24 h. Although the enhanced oxygen availability should theoretically trigger an increasing BC production rate, reduced yields have been also

previously reported [37]. According to Aydin and Aksoy [12], the high shear stress suffered by bacterial cells with high agitation and aeration triggers a spontaneous mutation of bacteria of the genus *Komagataeibacter* which deactivates the essential enzymes involved in the cellulose production, i.e. phosphoglucomutase and uridine diphosphoglucose pyrophosphorylase [14]. As there is a wide oxygen availability in the smaller flasks, the probability of bacterial cells to mutate is higher, thus meaning a lower BC productivity and thus mechanical properties enhancement. This behavior described previously in the literature matches the better results obtained for TI values at 15% (1) than those at 15% (3), so it has been accepted.

Finally, at the dosage of 45% (3), only a slight improvement in TI (1.9%) was found after 6 h of culture. In this case, the high amount of residual nutrients present in the handsheets induces a high increment in the TI. In addition, the BC amount produced during culture was not high enough to achieve deserving increments, probably also masked by the reinforcing effect of fructose in handsheets. Moreover, even a worsening in mechanical properties was observed for culture time longer than 12 h. The consumption of nutrients by bacteria together with the bad handsheets formation due to the numerous clusters of BC, was the responsible of this decrement.

This behavior agreed with the proposed mechanism of in situ culture for agitated systems, without observing any improvement in mechanical properties when culture time was higher than 24 h. Interestingly, when culture was carried out in just one flask, either 100 mL or 300 mL, TI results presented a similar behavior, increasing at the first stage, getting a maximum at 12 h of culture, and decreasing after that time. However, when both dosages were triplicated, TI values did not follow the same tendency at all, but increased at 6 h and decreased after that time.

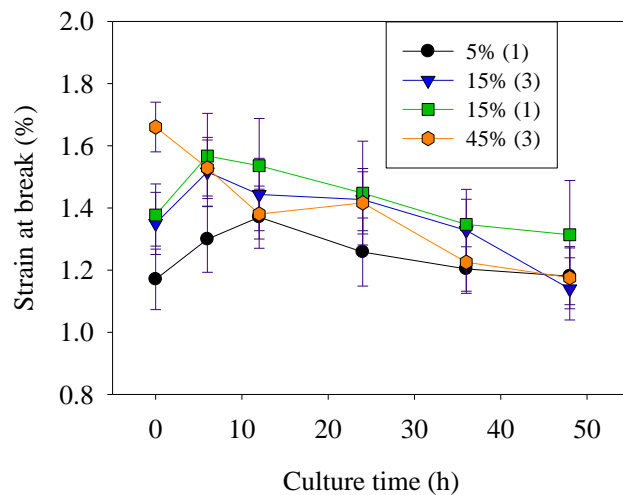


**Figure 8.** Tensile index of handsheets prepared with 5% (1), 15% (3), 15% (1) and 45% (3) of in situ cultured bacteria.

Strain at break was also increased at high dosages of in-situ cultured pulp at zero time (Figure 9). Thus, when the dosage was 5%, it was decreased by 9.3%, while it was increased by 4.6% and 28.7% at the dosages of 15% and 45%. As TI results, strain at break increased due to the higher number of hydrogen bonds between several units of fructose and cellulose. In this way, tensile strength was increased as well as the flexibility of the handsheets, as previously described [30].

The variation of the strain at break values with time at the dosage of 5% (1) matched with the TI behavior. At 6 h of culture, it increased by 11.1%, getting a maximum in 12 h with an improvement of 17.9%. However, when a 15% (3) of cultured pulp was added to the pulp, a maximum increment of 11.8% was found at 6 h of culture, decreasing after that time. Compared to 15% (1), while TI was highly improved, strain at break was almost similar. Then, the BC produced could be the driver for the enhancement in TI, but not for the strain at break improvement. In addition, the high error bars show low confidence data. Finally, at a high dosage of 45% (3), while strain at break at zero time was further

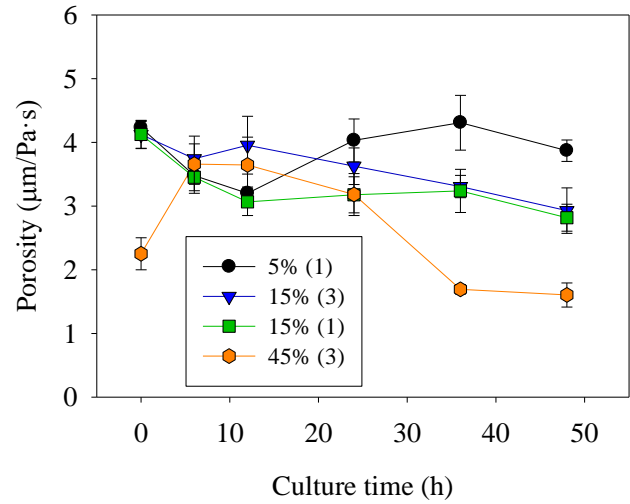
improved, it was decreased with the culture time, what is explained by the higher amount of BC and the nutrients consumption. Then, while TI results were so dependent on the BC production, strain at break seems to be affected in a high extent by the amount of residual nutrients in the handsheets.



**Figure 9.** Strain at break of handsheets prepared with 5% (1), 15% (3), 15% (1) and 45% (3) of pulp in-situ cultured by bacteria.

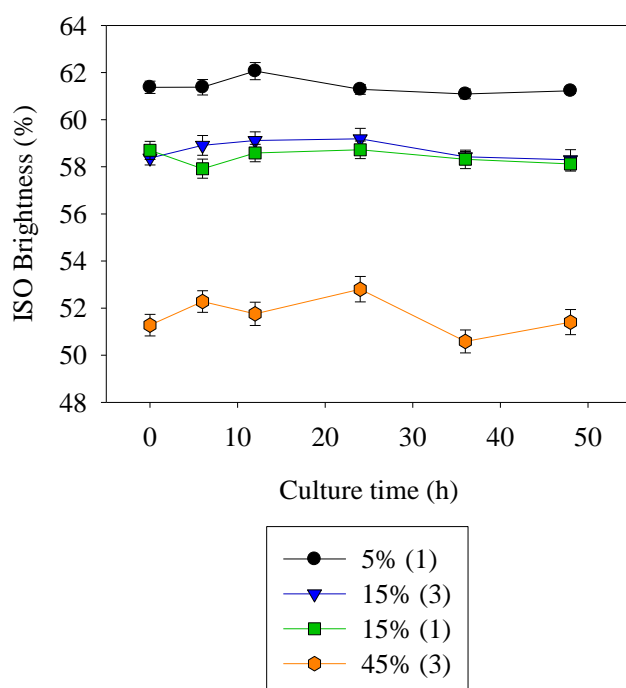
Porosity of handsheets was traditionally found to decrease by the addition of different nanocelluloses, since they fit the gaps between fibers [3]. Therefore, it was expected this parameter to be reduced with the culture time while the BC production is developed. In addition, as fructose is bonded to fibers by hydrogen bonds, and both TI and strain at break were increased by this residual component, porosity should be reduced. This is indeed what happened according to Figure 10, where increments of -5.2% and -49.4% were found for dosages of 15% and 45% at zero time, respectively. This explanation also agrees with the results observed for 5% (1), 15% (3) and 15% (1) with time. However, results for 45% (3) differ from the previous results. While TI slightly increased during the first 6 h of culture, porosity also increased by 62.7%. Then, it strongly decreased after

24 h of culture, until values were below the one of zero time (decrement of 28.8%). The reason for that behavior could be the ease of the nutrients to be retained within the BC network, forming an impermeable area.



**Figure 10.** Porosity of handsheets prepared with 5% (1), 15% (3), 15% (1) and 45% (3) of pulp in-situ cultured by bacteria.

Finally, Figure 11 showed a strongly dependence of the dosage with the optical properties and not with culture time (Figure 11). Then, when the dosage was 5% (1), the ISO brightness was 61.4%, being reduced to 58.3% and 52.0% for the dosages of 15% and 45%.



**Figure 11.** ISO Brightness of handsheets prepared with 5% (1), 15% (3), 15% (1) and 45% (3) of pulp in-situ cultured by bacteria.

Due to the possibility that some BC nanofibers can be loss during the removal of the medium components because of their small size, washing of the pulp has been avoided. However, this issue would become a drawback for large scale fabrication, not only affecting paper properties, but also incrementing the microbiological population in the wastewater as well as easing the presence of deposits in some papermaking equipment. Hence, further studies would, therefore, be necessary to overcome this challenge before scaling-up this method.

#### 4. CONCLUSIONS

The viability of the in situ culture of bacteria of the genus *Komagataeibacter* with recycled fibers to reinforce paper has been demonstrated. The presence of BC was verified in all experiments, being observed earlier in static culture.

In this latter case, BC was produced as a thick membrane floating on top of the layer of sedimented primary fibers, thus failing to improve the properties of paper. In agitated cultures, the production of BC appeared to present two stages: first, BC was found to cover the primary fibers, compensating thus for the fiber damage suffered during the recycling process; at a later time, these coated fibers were interwoven forming tight clusters difficult to disperse and producing heterogeneous papers. Agitated cultures showed capacity to improve mechanical properties of paper, but only in the first stage.

The BC production in agitated media in the first stage enhanced paper flexibility, which is not obtained using the traditional CNF or CNC. A maximum improvement in the mechanical properties was obtained at 12 h for the addition of 15% (1), reaching increments of 24.3% in TI and 19.4% in strain at break. Owing to the different mechanisms and improvement percentages, culture time was found a key parameter to be optimized.

Probably due to the combination of the correct hydrodynamic environment and culture volume, the dissolved oxygen concentration in the cultures with higher volume was suitable to avoid the shear stress and the diversion metabolic pathways towards the deactivation of the essential enzymes involved in the cellulose production. This shear stress was suffered by bacteria in cultures with small volume, thus reducing the BC productivity.

In view of the results, the in-situ BC production with recycled fibers constitutes a promising alternative to replace conventional strengthening agents. These results have the potential to be applied in industrial paper mills, employing pulp streams with the application of low cost, non-exhaustive sterilization operations, such as ozone or ultraviolet radiation. Further studies must be carried out to substitute nutrients by waste streams and thus reduce the costs.

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