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

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8 **ABSTRACT**

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

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

27 **1. INTRODUCTION**

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

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

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

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

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

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

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

79 **2. MATERIALS AND METHODS**

80 **2.1 Materials**

81 The bacterial strain *Komagataeibacter sucrofermentans* CECT 7291, used in this study,
82 was obtained from the Spanish Type Culture Collection (CECT). To retain the fibers, a
83 three-component retention system (C-F-B), commonly used in paper mills, was selected.
84 Polyamine (C) with a high molecular weight and a cationic charge density of 0.035 meq/g
85 was used as coagulant; polyacrylamide (F) with a high molecular weight and a cationic
86 charge density of 3.66 meq/g was used as flocculant; and hydrated bentonite clay (B) was
87 used as microparticle. These products were supplied by BASF (Ludwigshafen, Germany).
88 Recycled newsprint (NP) and magazine papers (MG) were kindly supplied by Holmen
89 Paper (Madrid, Spain). Nutrients, such as fructose, yeast extract and peptone, as well as
90 other reactants like NaCl, KCl, CaCl₂•2H₂O, NaHCO₃ and NaOH were of analytical
91 grade and supplied by Sigma-Aldrich.

92 **2.2 Bacterial growth and culture media preparation**

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

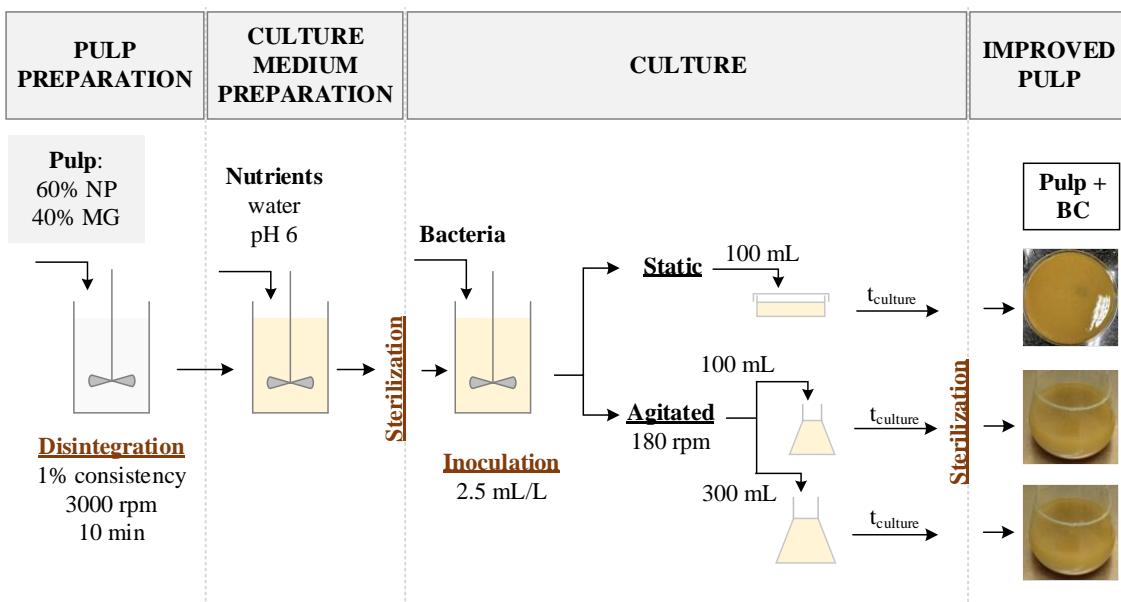
96 A pulp suspension of 0.5% consistency was prepared by pulping 60% NP and 40% MG
97 papers using a Messmer pulp disintegrator (Mavis Engineering Ltd, London, UK) at 3000
98 rpm for 10 min [3]. Then, 20 g/L fructose, 5 g/L yeast extract and 3 g/L peptone were
99 added to the pulp suspension and pH was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

100 The prepared suspension was autoclaved at 121 °C. Once it reached room temperature,
101 2.5 mL of the bacterial suspension were added to inoculate each liter of pulp.

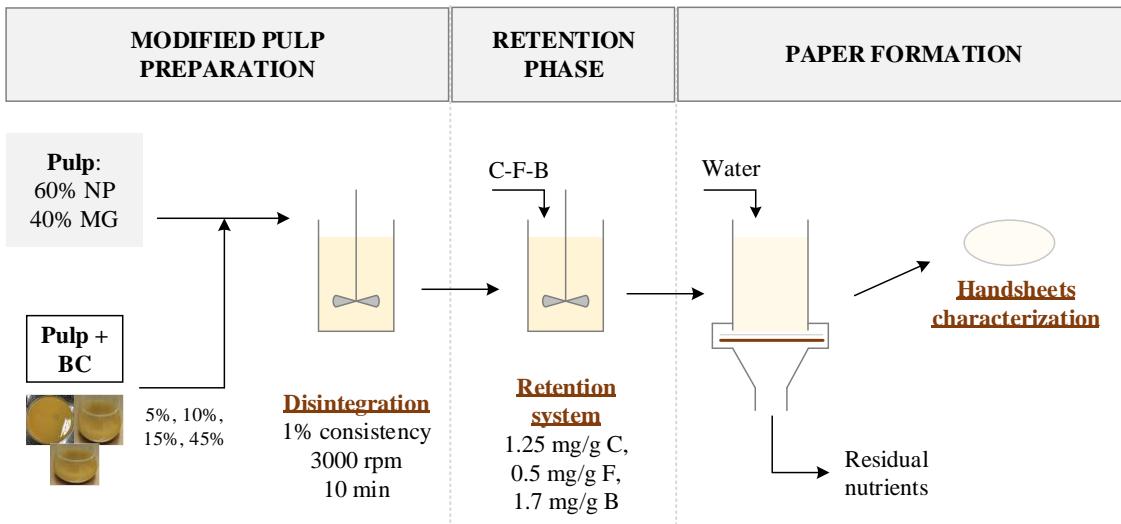
102 ***2.3 In-situ culture***

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

a)



b)



111

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

115 **2.4 Handsheets formation**

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

120 the mixture was pulped at 3000 rpm for 10 minutes. The retention system, (1.25 mg/g of
121 C, 0.5 mg/g of F and 1.7 mg/g of B) was added previously to handsheet formation. Then,
122 handsheets with a basis weight of 60 g/m² (ISO 5269/2 (2004)) were prepared with a
123 normalized Rapid-Köthen handsheet former (PTI, Vorchdorf, Austria). A schematic view
124 of the procedure is shown in Figure 1b.

125 **Table 1.** Used nomenclature for each experiment, corresponding to different amounts of
126 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

127 **2.5 Handsheet characterization**

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

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

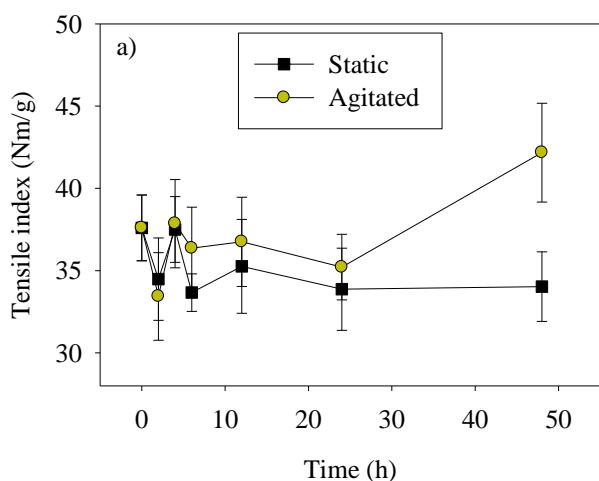
138 **3. RESULTS AND DISCUSSION**

139 ***3.1 Effect of culture mode on paper properties***

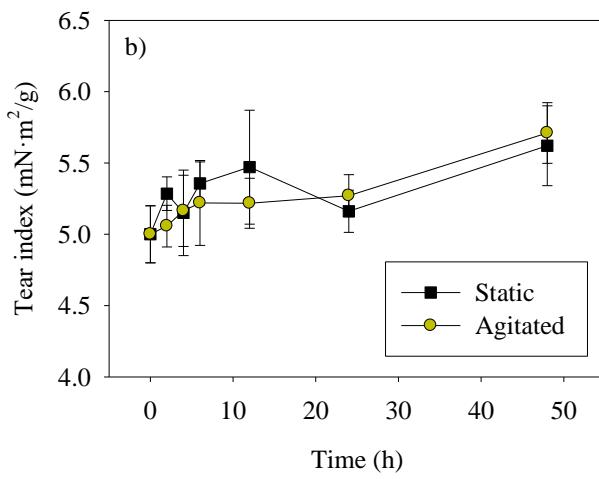
140 ***3.1.1 Mechanical properties***

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

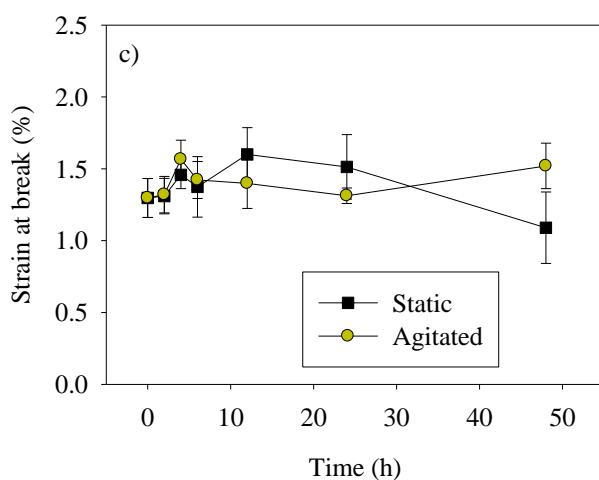
150 At 48h of incubation, TI of handsheets increased up to 42.2 Nm/g for agitated culture,
151 meaning an increase of 12.2%. At this time, the number of cell colonies keeps constant,
152 thus increasing the BC productivity. However, this is not as positive for static cultures,
153 where TI remained below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



158



159



160

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

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

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

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

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

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

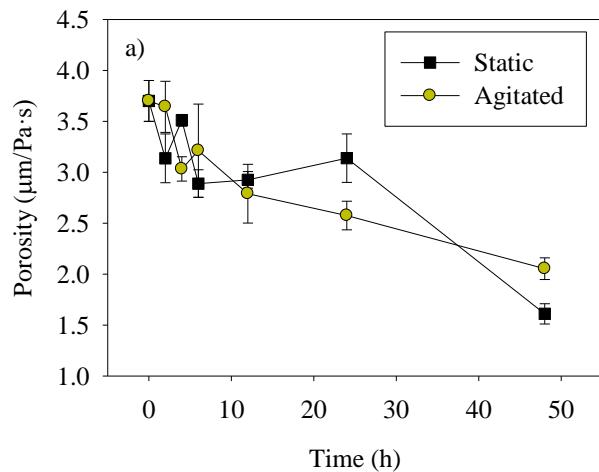
186 Strain at break results are represented in Figure 2c. When BC was produced in static
187 culture, strain at break increased by 24.1% the first 12 h, but it was reduced by 15.5%
188 after 48 h of culture compared to the initial value. This fact could be related to the irregular
189 formation of the handsheets, since the synthetized BC pellicles were difficult to disperse
190 [30]. However, in agitated culture, strain at break increased by 8.5%, when time was
191 below 24h, and by 17.8% at 48h, with also agrees with the higher flexibility found based
192 on TI and tear index results.

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

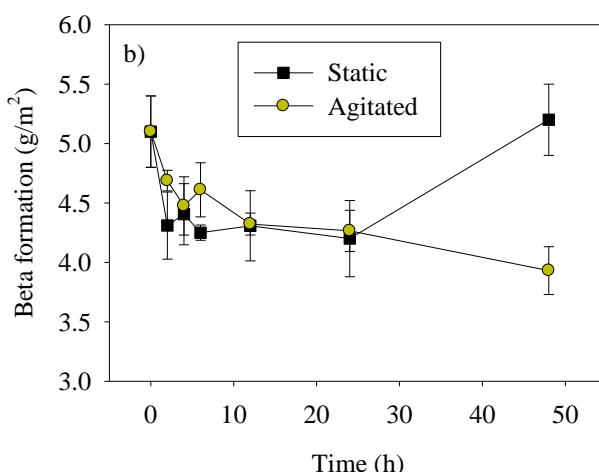
205 *3.1.2 Physical properties*

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

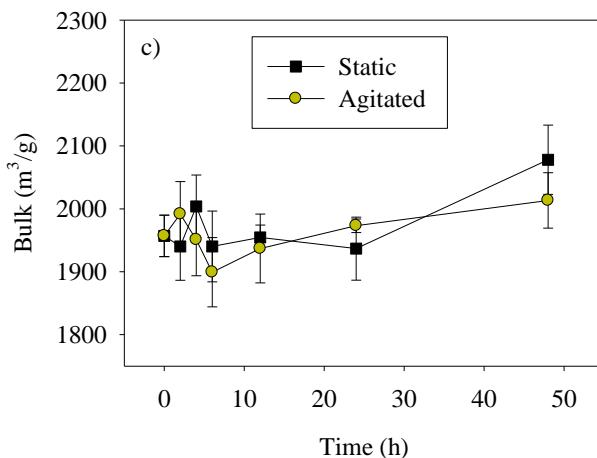
208 barrier properties [34]. As shown in Figure 3a, porosity of handsheets formed by culturing
209 the pulp in static mode presented a decreasing tendency with culture time. However,
210 porosity decreased in a smoother way in the case of agitated cultures, reaching a
211 decrement of 44.6% when culture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



212



213



214

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

217 Beta formation measurement shows the homogeneity of the handsheets as the standard
218 deviation of the microgrammage measured in different points of the sample. According
219 to Figure 3b, formation of handsheets prepared with the static cultured improved pulp
220 was more homogeneous when time was below 24 h, since beta formation decreased by
221 15.7%. However, the formation of a pellicle of BCNF during culture at high culture time
222 (48 h) triggered more irregular papers, increasing the beta formation index by 2%
223 compared to the initial sample.

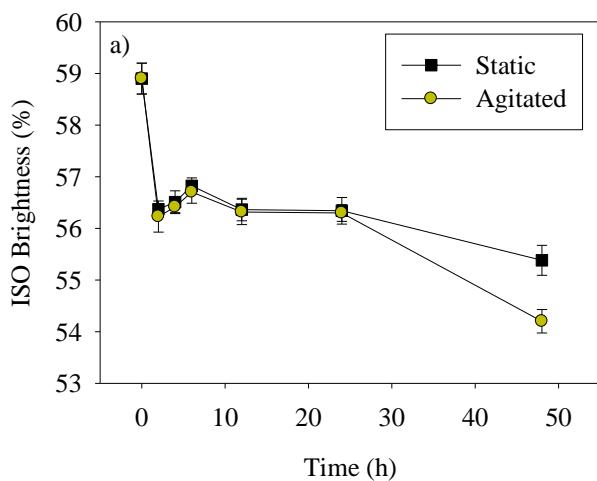
224 In the case of agitated cultures, beta formation presented a smoother decreasing tendency,
225 meaning that the production of BC was carried out homogeneously within the pulp. Even
226 at low culture times, below 6 h, this parameter was reduced by 12.2%, and by 20% at 48
227 h. These results for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

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

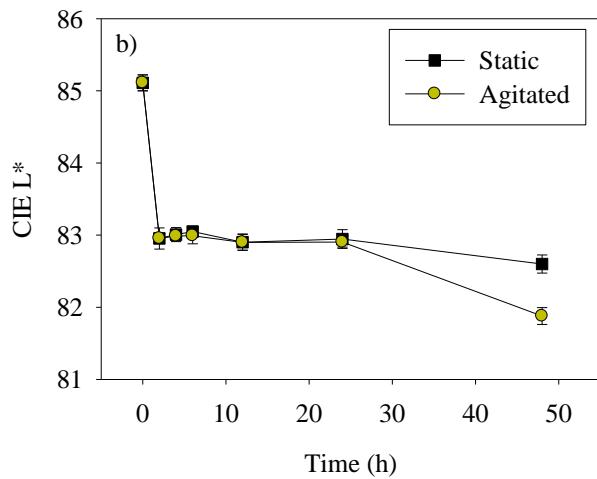
232 behavior was observed between both static and agitated cultures, decreasing the value of
233 this parameter at short times followed by an increase after 12 h. The first reduction was
234 assigned to the replacement of the original fibers by improved fibers of either BC alone
235 (static mode) or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

241 *3.1.3 Optical properties*

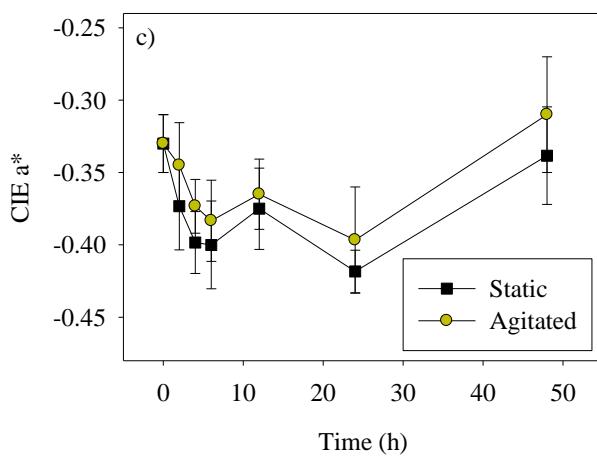
242 Optical properties were also affected by the addition of improved fibers to the pulp,
243 reducing the ISO Brightness of handsheets when the pulp was cultured even for short
244 times (Figure 4a). This effect could be assigned to the presence of nutrients in the
245 handsheets, some of them of brown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



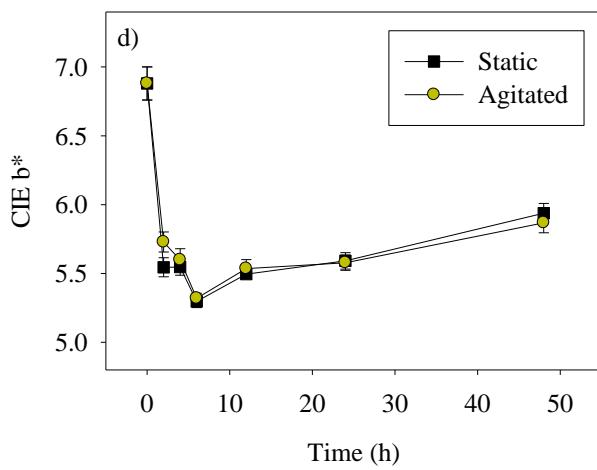
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257 **Figure 4.** Effect of culture time on the optical properties of paper in both static and
258 agitated mode: a) ISO Brightness, b) CIE L*, c) CIA a* and d) CIE b*.

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

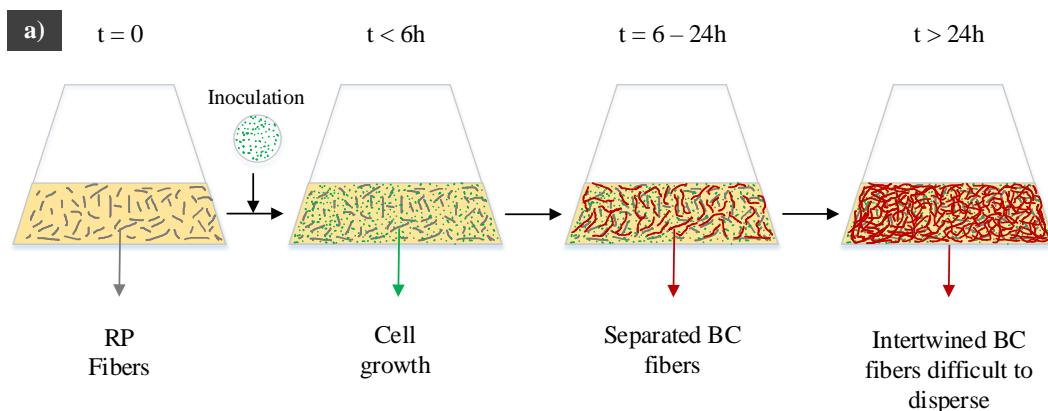
267 **3.2 Static and agitated culture: production mechanism**

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

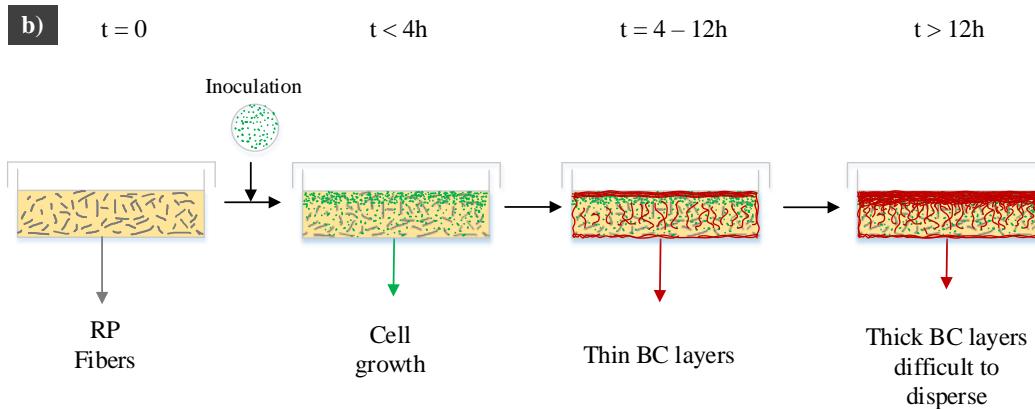
270 In the case of agitated culture (Figure 5a), RP fibers are distributed homogeneously
271 through the culture broth at zero time, as observed in Figure 6a, where a macroscopic
272 view of the culture broth is displayed. After the pulp was inoculated, mainly cell growth

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

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

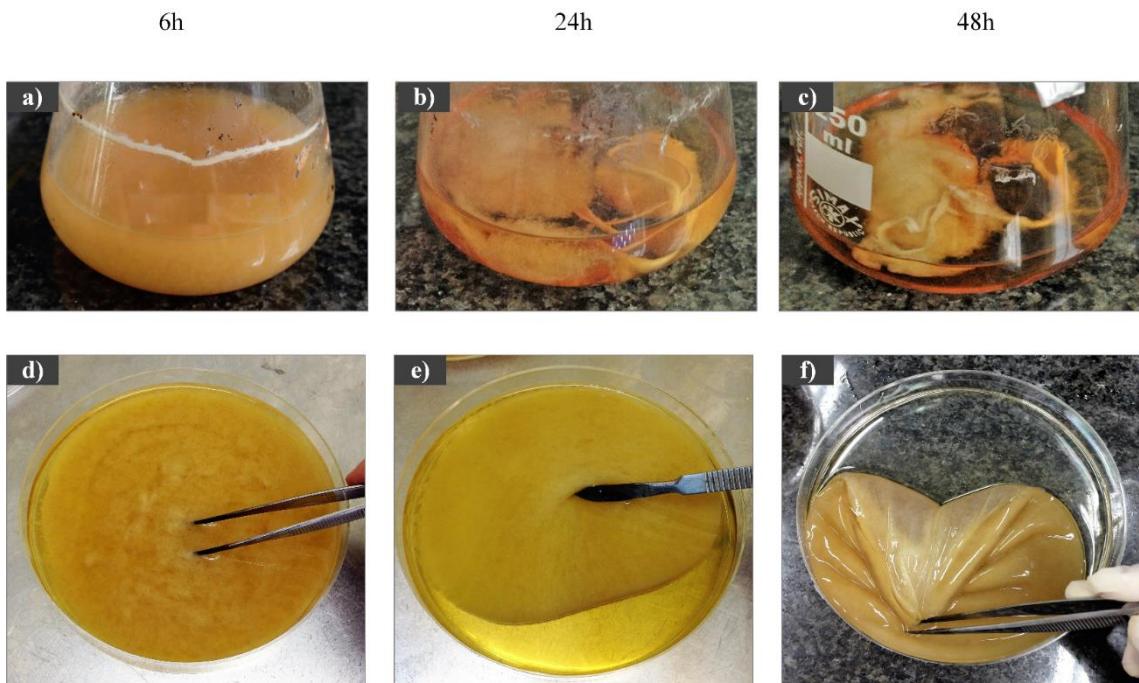


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292 **Figure 5.** Proposed mechanism for in-situ culture of bacteria with cellulosic fibers in a)
293 agitated mode and b) static mode.



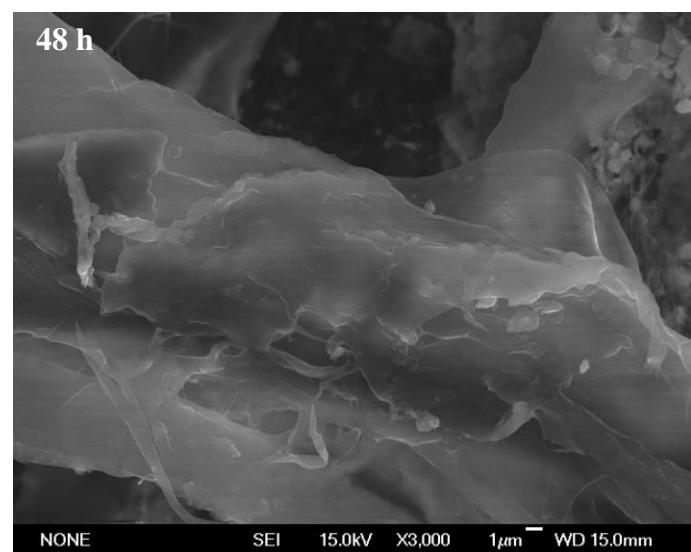
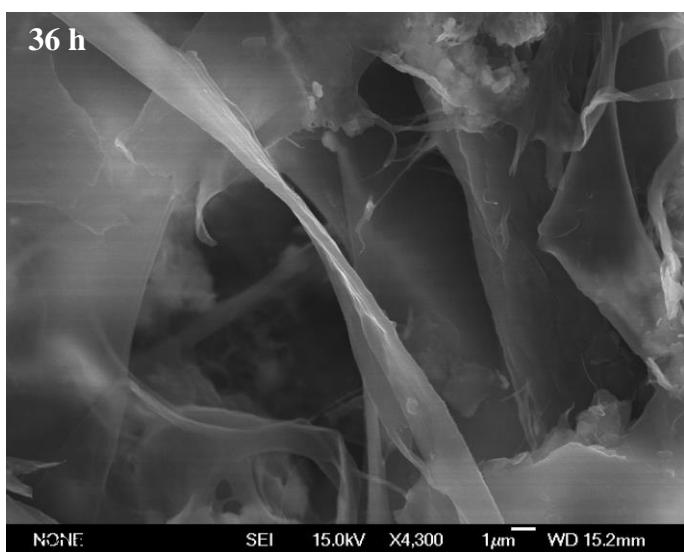
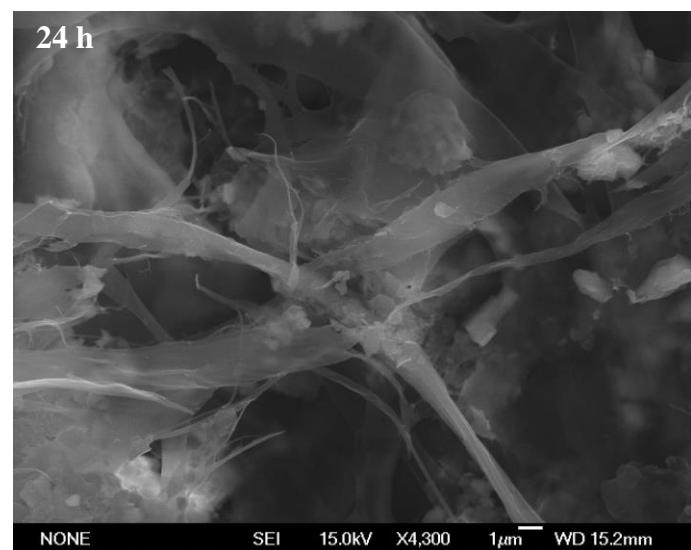
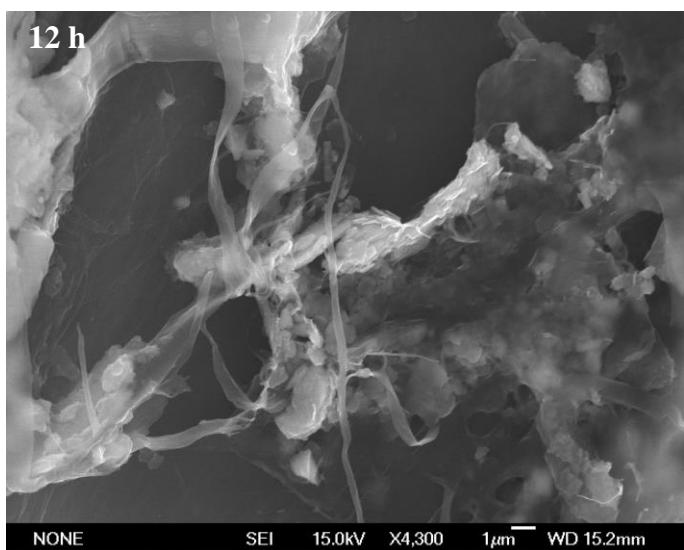
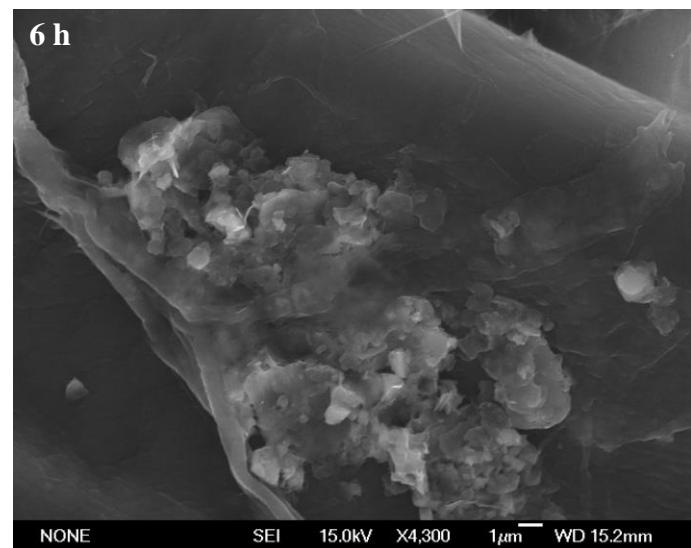
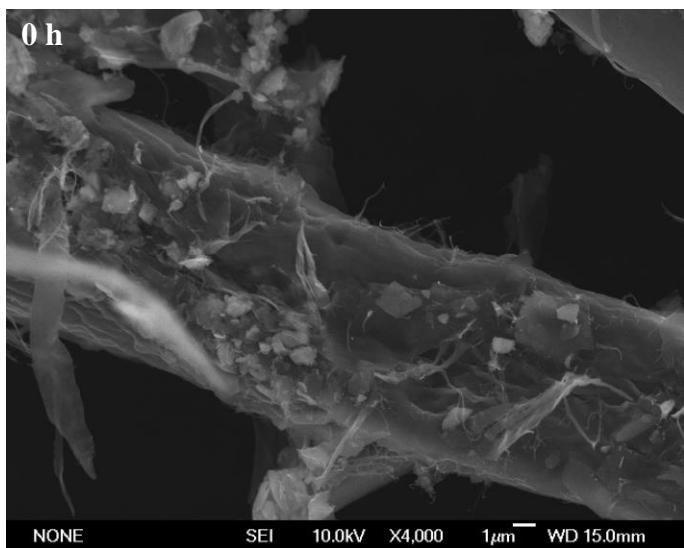
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295 **Figure 6.** Images of agitated (a, b and c) and static (d, e and f) in-situ cultures after 6 (a
296 and d), 24 (b and e) and 48 h (c and f) of culture time.

297 In the case of static culture, while recycled fibers tended to sediment, bacteria moved to
298 the surface of the culture broth in search of oxygen (Figure 5b). In this case, as published
299 previously [11], cell growth is slower, but as most of bacteria are concentrated on the
300 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.



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

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

335 ***3.3 Effect of the culture volume on paper properties***

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

340 Zero time values showed in Figure 8 were those obtained with the addition of cultured
341 pulp without inoculation at the different dosages. They show the tendency of TI to
342 improve with a higher dosage of the cultured pulp. Thus, for the dosages of 5%, 15% and
343 45%, TI increased by 8.5%, 15.3% and 28.4% compared to handsheets made of pulp
344 without nutrients. Fructose, the carbon source used in this study, is surrounded by
345 hydroxyl groups, which makes possible the bond between them and the cellulose chains

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

348 When the cultured pulp dosage was 5% (1), the handsheets TI was found to increase with
349 the culture time for the first hours, reaching improvements of 6.8% and 10.8% at 6 and
350 12 h. After that, TI decreased due to the heterogeneous formation of the handsheets, which
351 concentrated the improved fibers with BC in a specific area of the handsheets. This
352 behavior was related to the BC production mechanism in agitated culture previously
353 explained, since the improvement in mechanical properties was deceased after 24 h.

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

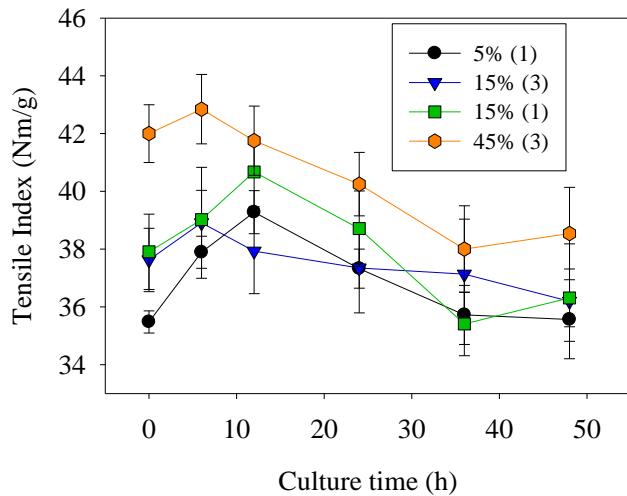
360 Nevertheless, when the same volume was cultured in one single flask (15% (1)), the
361 results were more satisfactory. The highest TI value was obtained at 12 h (40.7 Nm/g),
362 reaching an increment of 7.4% compared to the TI for zero time at this dosage. Then,
363 after 24 h of culture, the TI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

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

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

379 Finally, at the dosage of 45% (3), only a slight improvement in TI (1.9%) was found after
380 6 h of culture. In this case, the high amount of residual nutrients present in the handsheets
381 induces a high increment in the TI. In addition, the BC amount produced during culture
382 was not high enough to achieve deserving increments, probably also masked by the
383 reinforcing effect of fructose in handsheets. Moreover, even a worsening in mechanical
384 properties was observed for culture time longer than 12 h. The consumption of nutrients
385 by bacteria together with the bad handsheets formation due to the numerous clusters of
386 BC, was the responsible of this decrement.

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



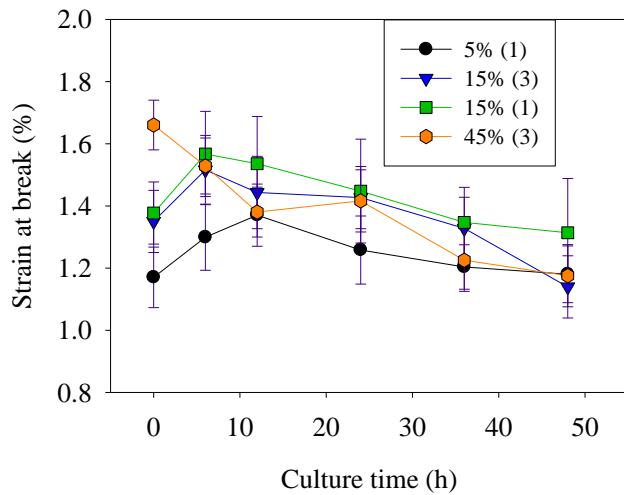
394

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

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

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

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

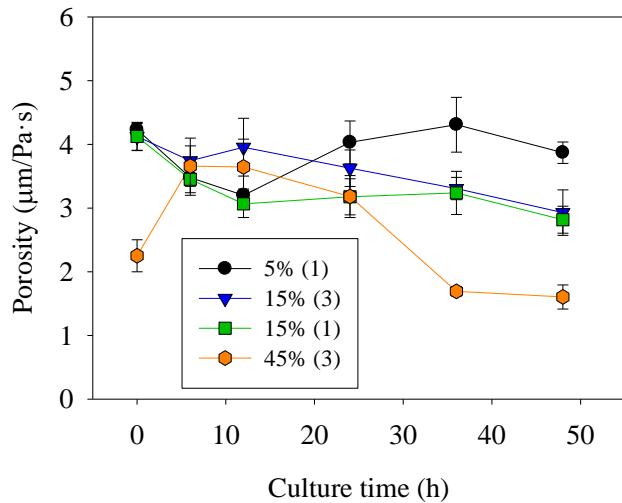


415

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

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

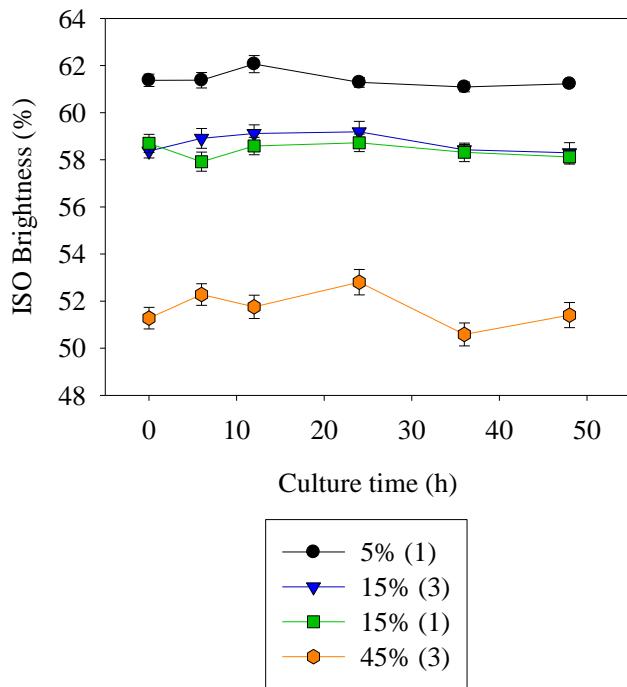
428 24 h of culture, until values were below the one of zero time (decrement of 28.8%). The
429 reason for that behavior could be the ease of the nutrients to be retained within the BC
430 network, forming an impermeable area.



431

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

434 Finally, Figure 11 showed a strongly dependence of the dosage with the optical properties
435 and not with culture time (Figure 11). Then, when the dosage was 5% (1), the ISO
436 brightness was 61.4%, being reduced to 58.3% and 52.0% for the dosages of 15% and
437 45%.



438

439 **Figure 11.** ISO Brightness of handsheets prepared with 5% (1), 15% (3), 15% (1) and
440 45% (3) of pulp in-situ cultured by bacteria.

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

448 **4. CONCLUSIONS**

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

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

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

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

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

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