The Effect of Carbon and Nitrogen Sources on Bacterial Cellulose Production and Properties from *Gluconacetobacter sucrofermentans* CECT 7291 Focused on its use in Degraded Paper Restoration

Sara M. Santos, José M. Carbajo, and Juan C. Villar *

Bacterial cellulose (BC) synthesized by *Gluconacetobacter sucrofermentans* has a high degree of crystallinity, durability, great resistance, and biocompatibility. This study evaluates the effect that carbon and nitrogen sources, present in HS (Hestrin–Schramm) culture medium, have on BC from *G. sucrofermentans* CECT 7291 used to restore damaged documents. The initial pH of the medium and the temperature were set for all assays, which were conducted in static conditions. The cellulose layers were obtained at four time points. The growth media were characterized at each time point (pH and carbon source consumption). Cellulose layers were washed, pressed, dried, and characterized by determining their pH, dry weight, and optical and mechanical properties. The best combination of carbon and nitrogen sources proved to be fructose plus yeast extract–corn steep liquor, with or without ethanol, which provided a good balance between BC production and carbon source consumption, and generated a resistant and homogeneous cellulose layer.

Keywords: Bacterial cellulose; Gluconacetobacter; Carbon source; Nitrogen source; Paper restoration

Contact information: INIA-CIFOR, Ctra. de La Coruña km 7.5, 28040 Madrid, Spain;
**Corresponding author: villar@inia.es*

INTRODUCTION

Cellulose is the most abundant biopolymer on earth. It is produced by a great variety of organisms, including vascular plants, marine algae, and prokaryotic organisms. Bacterial cellulose (BC) is an insoluble exopolysaccharide generated by bacterial species, for example those of the genera *Acetobacter*, *Agrobacterium*, and *Aerobacter* (Shoda and Sugano 2005).

The genus *Acetobacter* refers to a group of bacteria that have the ability to oxidize sugars, sugar alcohols, and ethanol, producing acetic acid as the major end product. In particular, *A. xylinum* effectively synthesizes cellulose as a primary metabolic product. *A. xylinum* was reclassified as *Gluconacetobacter xylinus* and assigned to the novel genus *Gluconacetobacter* (Yamada *et al*. 1997), which also includes *G. hansenii*, *G. europaeus*, *G. oboediens*, and *G. intermedius*. *G. xylinus* BPR2001 is the only strain of the subspecies *G. xylinus* subsp. *sucrofermentans*. Selected for its ability to produce cellulose (Toyosaki *et al*. 1995), this bacterial strain has been recently reclassified as a separate species and named *Gluconacetobacter sucrofermentans* (Cleenwerck *et al*. 2010).

G. sucrofermentans is a rod-shaped, obligate aerobic, gram-negative bacterium (Kersters *et al*. 2006). BC synthesized by *G. sucrofermentans* has specific physical and chemical properties that distinguish it from plant cellulose, such as high crystallinity

(more than 60%), chemical purity, mechanical strength, high water-holding capacity, and biocompatibility (Ross *et al*. 1991). For example, when compared to plant cellulose, BC ribbons are only one-hundredth in width (Shoda and Sugano 2005) and two orders of magnitude higher in tensile strength (Yamanaka *et al*. 1989).

BC synthesis occurs as a multi-step series of chemical reactions beginning with the incorporation of monomeric glucose into a polyglucosan (Jonas and Farah 1998). A single bacterial cell may polymerize up to 200,000 molecules of glucose per second into β-1,4-glucan chains (Hestrin and Schramm 1954) that aggregate into subfibrils. These subfibrils are extruded through pores arranged on the cell surface forming a row along the longitudinal axis. Approximately 10–100 subfibrils aggregate to form crystalline microfibrils about 3.5 nm in diameter (Jonas and Farah 1998). Subsequently, these microfibrils are gathered into bundles, which group to form ribbons (Yamanaka *et al*. 2000). These ribbons interact with ribbons from other cells, forming a two-dimensional layer. Finally, parallel layers interact with one another by hydrogen bonds and Van der Waals forces, forming a gelatinous suspension in the liquid medium (Brett 2000). Initially, each BC layer contains absorbed water that can be removed, leaving chemical groups available for the creation of new hydrogen bonds between adjacent cellulose chains, a process that increases crystallinity (Colvin and Leppard 1977).

The macroscopic morphology of BC depends on the culture conditions (Yamanaka *et al*. 2000). In a static culture, a gelatinous membrane of cellulose fibers is formed (Czaja *et al*. 2004). The result is high mechanical strength and crystallinity (60% to 80%). However, in an agitated culture, cellulose mats interconnect less frequently, forming irregular granules (Vandamme *et al*. 1998).

In their natural habitat, *Gluconacetobacter* species grow in fruits, flowers, and degraded food. These bacteria live entrapped in a mesh of cellulose, synthesized by themselves. This cellulose mesh keeps the organisms in close contact with oxygen from the air and also fixed to the substrate (Hestrin and Schramm 1954; Williams and Cannon 1989). Okamoto *et al*. (1994) suggested that BC actually plays a role as a food reservoir and can be consumed by the microorganisms if other nutrients get scarce.

Thanks to its unique properties, BC has multiple applications in fields as diverse as the paper, textile, and food industries, and is also used as a biomaterial for manufacturing cosmetics, artificial skin, artificial blood vessels, and high-fidelity speakers (Chawla *et al*. 2009). Its application in the restoration of old paper documents has apparently not been sufficiently explored yet, although its biocompatibility and exceptional mechanical properties make BC an optimal candidate for this purpose.

Several studies have focused on the mechanism of synthesis of this biopolymer, as well as on its structure and properties (Hestrin and Schramm 1954; Kai and Keshk 1999). The most relevant culture parameters for BC production are the cultivation method (static or agitated), the carbon and nitrogen sources, the pH, and the temperature (Chawla *et al*. 2009). Those studies that have evaluated different pH settings have shown that optimum values range from 4.5 to 7.5 with the greatest efficacy being located around 6.5 (Son *et al*. 2001; Çoban and Biyik 2011). The optimum temperature for BC production is 30 ºC (Son *et al*. 2001; Pourramezan *et al*. 2009).

Efficient cellulose production by this bacterium lies in its ability to synthesize glucose from the carbon substrate, followed by its polymerization to cellulose. Different carbon sources, such as mono- and oligosaccharides, alcohols, and acids have been tested to maximize BC production by several *G. xylinum* strains (Chawla *et al*. 2009). For instance, cellulose yields have been the highest when using sucrose and glycerol as

carbon substrates for the *G. xylinus* strain ATCC 53524 (Mikkelsen *et al*. 2009); sucrose also gave the best result with *Acetobacter* sp. 4B-2 (Pourramezan *et al*. 2009); glycerol was the best substrate for *G. xylinus* ATCC 10245 (Keshk and Sameshima 2005); and glucose was found to be the best carbon source for *Acetobacter lovaniensis* HBB5 (Çoban and Biyik 2011). Cellulose yields are also maximized with particular sources of nitrogen. Which source of nitrogen provides the highest cellulose yield varies depending on the bacterial strain that is to be used. For example, Çoban and Biyik (2011) selected yeast extract for BC production with *Acetobacter lovaniensis* HBB5, while Rani and Appaiah (2011) found that corn steep liquor (CSL) is an effective nitrogen source for *G. hansenii* UAC09. The same nitrogen source, CSL, was the most suitable one for BC production with *G. sucrofermentans* (Tsuchida and Yoshinaga 1997). Only a few reports evaluated the combined effect that the selection of carbon and nitrogen sources has on BC production by *A. xylinum*, although Ramana *et al*. (2000) proposed different combinations that efficiently produce BC.

Naritomi *et al*. (1998) observed that the supplementation of 1% ethanol to a medium containing 30 g/L of fructose increases BC production by *G. sucrofermentans* BPR3001A. This study also suggests that ethanol acts not as a substrate for BC biosynthesis but as an energy source for ATP generation in the viable cells, increasing BC production as a result. Similar results have been found in studies with *G. hansenii*, (Park *et al*. 2003) and with *A. xylinum* ATCC 10245 (Yunoki *et al*. 2004). In the latter report, the authors suggest that ethanol was not the carbon source for BC production and that the improvement was a consequence of ethanol suppressing the transformation of glucose in gluconic acids, a process that potentiates a more efficient polymerization of glucose into BC. However, if the ethanol percentage is increased over 1.5%, BC production decreases due to the inhibition of cell growth caused by the accumulation of acetate (Naritomi *et al*. 1998; Park *et al*. 2003).

All this evidence shows that there is no pattern of bacterial behavior in a given species that can lead to the selection of the most appropriate carbon and nitrogen sources for BC production. In these studies, the rate of BC production has been prioritized as the criterion for selecting growth conditions. As BC has shown promising results in areas as diverse as medicine and materials science (Chawla *et al*. 2009), quality criteria must also be considered in the selection of the most appropriate carbon and nitrogen sources. Thus, in the field of research of old paper restoration, there are parameters, such as mechanical strength, brightness, or yellowness of the BC, that are paramount when deciding whether or not to implement its use. Some studies have assessed the mechanical properties of paper made by mixing a low percentage of BC and vegetal cellulose (Surma-Ślusarska *et al*. 2008; Basta and El-Saied 2009; Gao *et al*. 2011), but there are almost no studies about the mechanical properties of paper made exclusively of BC (Yamanaka *et al*. 1989, Gea *et al*. 2011).

This project aims for the production of high-quality BC from *G. sucrofermentans* CECT 7291 to be used in the restoration of old papers. To achieve this goal, the objectives were the selection of carbon and nitrogen sources capable of providing cellulose pellicles with high strength and adequate brightness, and the study of the relationship between the physical properties of BC and the culture conditions.

EXPERIMENTAL

Microorganism and Culturing Maintenance

Gluconacetobacter sucrofermentans (previously known as *Gluconacetobacter xylinus* subsp. *sucrofermentans*) CECT 7291 was obtained from the Spanish Type Culture Collection (CECT). For maintenance it was subcultured periodically in HS medium (Hestrin and Schramm 1954) containing the following ingredients: glucose, 20 g/L; yeast extract, 5 g/L; peptone, 5 g/L; Na_2HPO_4, 2.7 g/L; and citric acid, 1.15 g/L. Agar was added in a final concentration of 15 g/L to solidify the culture media. Petri dishes were incubated at 30 ºC for 4 days and were kept at 4 ºC afterwards.

Culture Conditions

G. sucrofermentans was grown in HS solid media placed in Petri dishes for 6 days in order to obtain the suspension of bacterial cells to be used in the experiments. 500-mL Erlenmeyer flasks containing 100 mL of liquid HS media were inoculated with these plates and cultivated in static conditions for 4 days. Subsequently, the pellicles formed were cut in small pieces (about 1 × 1 cm) in sterile conditions and shaken with the liquid media at 700 rpm for 30 min. The suspension obtained was filtered through gauze, centrifuged at 4000 rpm for 10 min and, after removing the supernatant, the pellet was washed with Ringer's solution (NaCl, 2.5 g/L; KCl, 0.105 g/L; $CaCl_2 \cdot 2H_2O$, 0.120 g/L; and $NaHCO_3$, 0.05g/L). This solution was centrifuged again in the same conditions and the pellets were re-suspended in a small volume of Ringer's. The optical density of the solution was adjusted to 0.59–0.64 with a wavelength of 600 nm, diluting with Ringer's solution. An amount of 250 µL of the final solution was used to inoculate 100 mL of media.

For the carbon source experiments, glucose was substituted with fructose, sucrose, mannitol, or glycerol in the HS media, always at a final concentration of 20 g/L. For the nitrogen source experiments, HS media were prepared with the carbon source selected in the previous experiment; the original nitrogen sources (peptone and yeast extract) were replaced by different types of nitrogen sources: defined organic (asparagine), complex organic (yeast extract, CSL), ammonium inorganic (ammonium sulfate), and nitric inorganic (potassium nitrate). The combinations tested were: peptone–asparagine, yeast extract–asparagine, peptone–potassium nitrate, yeast extract–potassium nitrate, peptone–ammonium sulfate, yeast extract–ammonium sulfate, peptone–corn steep liquor, and yeast extract–corn steep liquor (CSL was obtained from Sigma-Aldrich, peptone and yeast extract from Cultimed). All these nitrogen sources were always added to obtain a final concentration of 5 g/L for each one of them. HS media, modified with the selected carbon and nitrogen sources, was supplemented with 1% ethanol to test its influence. Ethanol was sterilized by filtration and added in sterile conditions to the media.

In all cases, 100 mL of liquid medium was added to 150-mm Petri dishes, inoculated with the suspension described above, and cultivated at 30 ºC under static conditions. A total of 20 plates per source were analyzed after 4, 7, 10, and 13 days of cultivation, 5 plates each day.

Culture Media Characterization

At each condition and time point, the pH was determined in the residual culture medium. Also, the concentration of the residual carbon source was measured using specific enzymatic kits provided by Megazyme.

Cellulose Characterization

The cellulose pellicle was washed thoroughly with distilled water and its pH was measured using a surface electrode. To eliminate bacterial cells, the pellicles were incubated at 90 ºC in 1% NaOH for 30 min. Next, the pellicles were washed with distilled water again and the surface pH was measured using a pH-meter with a surface electrode. Cellulose films were prepared by filtering through a Buchner funnel of adequate diameter. For this purpose, a filter paper with a medium flow rate was used (PRAT DUMAS A150600), which subsequently was pressed at 2 kg/cm^2 for 1 h. Dry films were conditioned under standard conditions (23 ºC and 50% relative humidity) and characterized. This characterization consisted in determining their grammage (ISO 536), yellowness, opacity (ISO 2471), and burst (ISO 2758) and tear (ISO 1974) indexes, with 5 repetitions per grammage test, 20 per optical properties, 8 per burst index test, and 4 per tear index test at each time point.

RESULTS AND DISCUSSION

Carbon Source Selection

The effectiveness for cellulose production is shown in Fig. 1. The pattern of carbon source consumption changes depending on the source. Thus, glycerol and fructose are consumed slowly. On the other hand, glucose, sucrose, and mannitol cause a quick response; in these cases, after 7 days (2nd sampling) less than 20% of the initial carbon source remains.

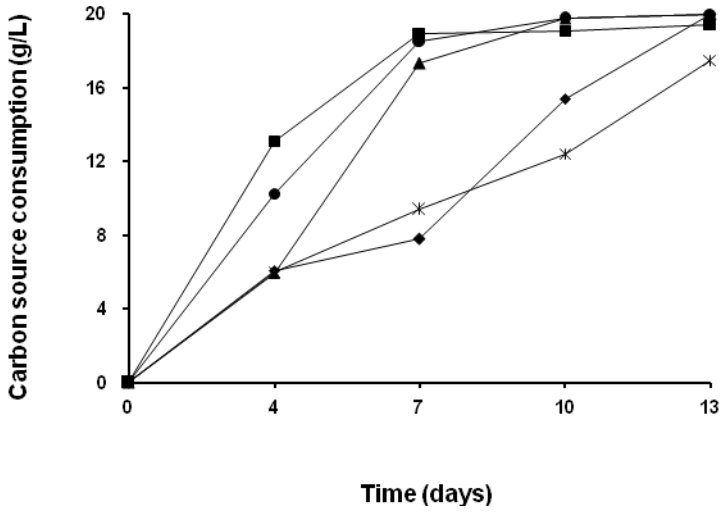

Fig. 1. Carbon source consumption. (♦) Glycerol, (■) Glucose, (▲) Sucrose, (●) Mannitol, and (×) Fructose

This pattern of consumption does not have a direct relationship to the production of cellulose (Fig. 2), as the efficiency in the utilization of the carbon source varies. With glucose and sucrose, a small amount of cellulose is produced with the maximum within a short time, while with glycerol, mannitol, and fructose, cellulose is produced consistently until the end of the test and in a greater amount than with glucose and sucrose. Different rates of production depending on the carbon source have already been described by Mikkelsen *et al*. (2009). These authors also observed that BC production started later with sucrose than with the other carbon sources. The onset delay for BC production with sucrose may be because it has to be hydrolyzed to glucose and fructose in the periplasm due to the impossibility of transporting sucrose through the cell membrane, (Velasco-Bedrán and López-Isunza 2007). Keshk and Sameshima (2005) studied glucose, glycerol, and fructose as carbon sources and obtained similar results regarding carbon source consumption but found a different behavior of BC production.

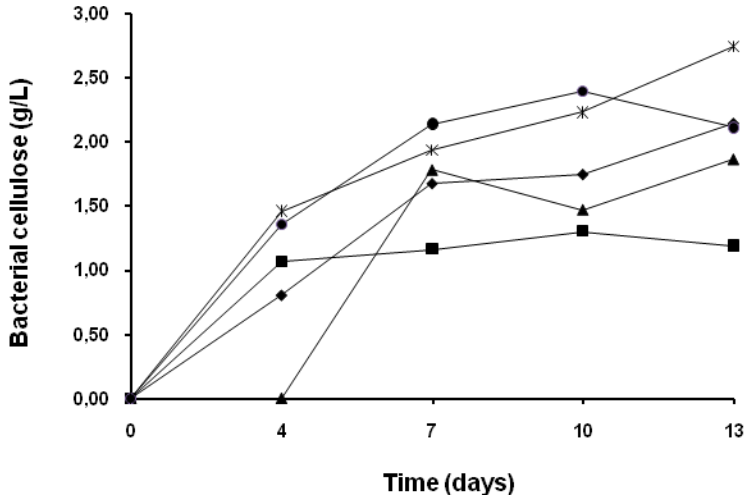

Fig. 2. BC production for different carbon sources. (♦) Glycerol, (■) Glucose, (▲) Sucrose, (●) Mannitol, and (×) Fructose

The differences observed among carbon sources may be a consequence of there being two major operative amphibolic pathways: the pentose phosphate cycle and the Krebs cycle, both for the oxidation of carbohydrates (Ross *et al*. 1991). At any rate, Mikkelsen *et al*. (2009) concluded that the products formed are indistinguishable, from the point of view of its molecular and microscopic characteristics, regardless of the carbon source that is used.

This study's results indicate that BC production improves when the carbon source for *G. sucrofermentans* is fructose, as a considerable amount of cellulose is produced (2.7 g/L) without exhausting the available carbon source.

Another interesting parameter to be considered when selecting the carbon source is the pH. If the BC is going to be generated directly on a material which could be degraded below a certain pH, as is the case with some ancient historical documents, an adequate pH must be obtained in order to avoid the acid hydrolysis of cellulose paper,

although *G. xylinum* tolerates acid and would be able to grow at a pH as low as 3.5. As Fig. 3 shows, in media with fructose, mannitol, and glycerol, the pH decreases less than a unit (final value 5.5–6.0), which is compatible with paper restoration. However, in the case of media with glucose and sucrose, the pH decreases below 4.5. These results are consistent with those of Keshk and Sameshima (2005). This rapid drop in pH is caused by the known conversion of glucose to gluconic acid (Hwang *et al*. 1999). Sucrose induces a similar decrease of the pH, although this effect is less pronounced because it dissociates into glucose and fructose, so the glucose available to convert into gluconic acid is just half the amount. After washing the BC pellicles with NaOH and distilled water, their pH, which originally was that of the growth media, becomes independent of the carbon source. Therefore, the pH of the BC pellicles is an issue only if cellulose is generated on the paper that is to be restored, but not if cellulose is collected from the bacterial growth media first and applied to the paper later. If the situation is the former, glucose and sucrose should not be used as carbon sources.

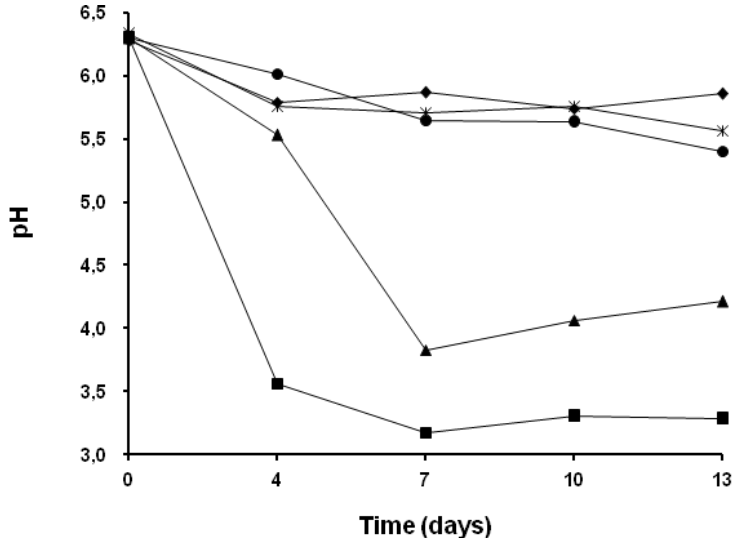

Fig. 3. Carbon source selection: Culture media pH. (♦) Glycerol, (■) Glucose, (▲) Sucrose, (●) Mannitol, and (×) Fructose

The mechanical and optical properties of BC are subject to variability (Table 1). Thus, the burst index varies between 5 and 15 kPa m^2/g with no apparent pattern. In all cases the values of this parameter were higher than those typically found in the paper industry. In the paper industry, the presence of more fiber–fiber bonds, which is achieved by refining, implies higher burst and tensile strength (Smook 1990). The presence of numerous fiber-fiber bonds in BC cellulose films comes from the very fine size of BC fibers that leads to their very high surface area available, hence the high values of burst index. The tear strength values are widely dispersed and always low regardless of the carbon source, which is consistent with findings reported by Surma-Ślusarska *et. al*. (2008).

The optical properties of BC (Table 1) also show a dispersed distribution, especially yellowness, which ranges from 10% to 40%, but with no clear trend. It should be borne in mind that the optical properties may subsequently be modified by various

treatments (bleaching, mineral addition, colorants, fluorescence whitening agents, *etc*.) to fit the final use of the BC.

Examining BC production rate and its properties enabled selection of the most appropriate carbon source to perform the second part of this study, which focused on the nitrogen source. It was always clear that all mechanical properties had high enough values, regardless of the carbon source, and that the optical properties were also acceptable. Therefore, the main criterion that the choice was based on was a high BC production rate at an adequate pH. This criterion sets fructose as the best carbon source. Consequently, fructose was used as the carbon source in the experiments described below, which were conducted to select the most appropriate nitrogen source.

Table 1. Variation of Optical and Mechanical Properties of BC Layers at Different Time Points Depending on the Carbon Source Used

Carbon Source	Time (days)	Burst Index (kPa m^2/g)	Tear Index (mN m^2/g)	Opacity (%)	Yellowness (%)
Glucose	4	7.60	2.10	20.85	12.80
	7	11.7	3.30	17.75	10.41
	10	10.2	1.50	21.75	25.15
	13	9.60	2.80	15.24	20.35
Fructose	4	10.4	0.90	20.13	20.17
	7	11.4	1.50	16.93	36.03
	10	6.60	0.60	71.41	39.27
	13	7.00	0.80	20.56	33.48
Sucrose	4	a	a	a	a
	7	6.70	1.10	12.67	14.89
	10	5.20	1.20	13.24	30.76
	13	11.0	1.80	17.56	29.96
Glycerol	4	b	2.20	15.13	2.41
	7	11.7	1.70	17.14	35.03
	10	14.9	1.70	24.43	30.51
	13	13.6	1.00	23.00	30.97
Mannitol	4	10.3	2.70	16.32	19.11
	7	13.5	1.60	15.83	38.41
	10	12.9	1.40	16.26	40.71
	13	12.0	0.90	18.62	14.26
a, BC layer not formed; b, under the sensibility limit					

Nitrogen Source Selection

Previous experiments on solid medium have shown that *G. sucrofermentans* CECT 7291 does not grow in the absence of organic nitrogen. This is consistent with the results that Rani and Appaiah (2011) report for *G. hansenii* UAC09 and Son *et al*. (2001) for *Acetobacter* sp. A9. Therefore, a source of organic nitrogen was included in all combinations: yeast extract–peptone (YE+P), yeast extract–asparagine (YE+Asn), yeast extract–potassium nitrate (YE+KNO_3), yeast extract-ammonium sulfate (YE+$(NH_4)_2SO_4$), yeast extract–corn steep liquor (YE+CSL), peptone–asparagine (P+Asn), peptone–potassium nitrate (P+KNO_3), peptone–ammonium sulfate (P+$(NH_4)_2SO_4$), and peptone–corn steep liquor (P+CSL).

At the end of the assay, the following combinations did not yield any BC: P+Asn, P+KNO_3, P+$(NH_4)_2SO_4$, and YE+$(NH_4)_2SO_4$. It was decided to proceed with these assays for a longer time. Only the combination YE+$(NH_4)_2SO_4$ ended up producing a cellulose

layer, but it was not used because of its very low yield. The rest of the fructose consumption and cellulose production data are shown in Fig. 4 and Fig. 5.

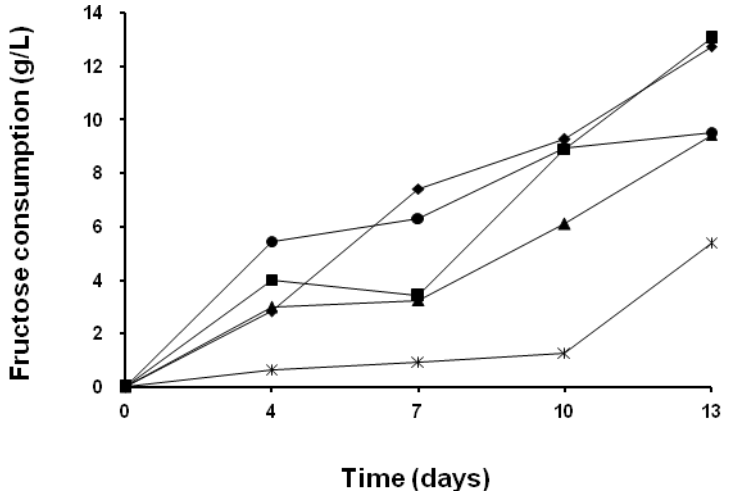

Fig. 4. Nitrogen source selection: Fructose consumption. (♦) YE+P, (■) YE+CSL, (▲) YE+Asn, (●) P+CSL, and (×) YE+KNO_3

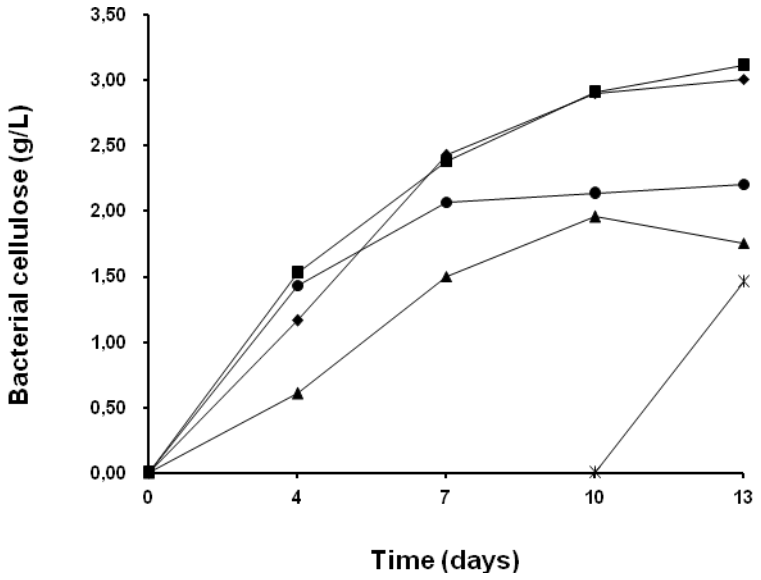

Fig. 5. Nitrogen source selection: BC production. (♦) YE+P, (■) YE+CSL, (▲) YE+Asn, (●) P+CSL, and (×) YE+KNO_3

Of all the tested combinations that produce BC, the only one in which an inorganic nitrogen source was used (YE+KNO_3) resulted in low fructose consumption and low cellulose production from day 10, which is an indication of low bacterial growth. With the use of asparagine, BC is obtained from the beginning of the test, but the maximum production is still low and never reaches 2.0 g/L. Peptone or corn steep liquor, in combination with yeast extract, provides the highest yield in BC production, with values around 3.0 g/L at the end of the experiments. However, the P+CSL combination produces a smaller amount of cellulose. These findings clearly establish the need to use

yeast extract in combination with peptone or corn steep liquor to obtain a high BC yield. All these combinations show a similar consumption of fructose. This is consistent with Çoban and Biyik (2011). Tsuchida and Yoshinaga (1997) observed that the presence of lactate in CSL stimulated cell growth and BC production by *G. sucrofermentans*.

BC production always develops at a moderate pH ranging from 5.2 to 6.4, and only the medium with YE+Asn seems to have a rising trend beyond this range (Fig. 6). This could be due to the presence of ammonium from asparagine degradation in the medium.

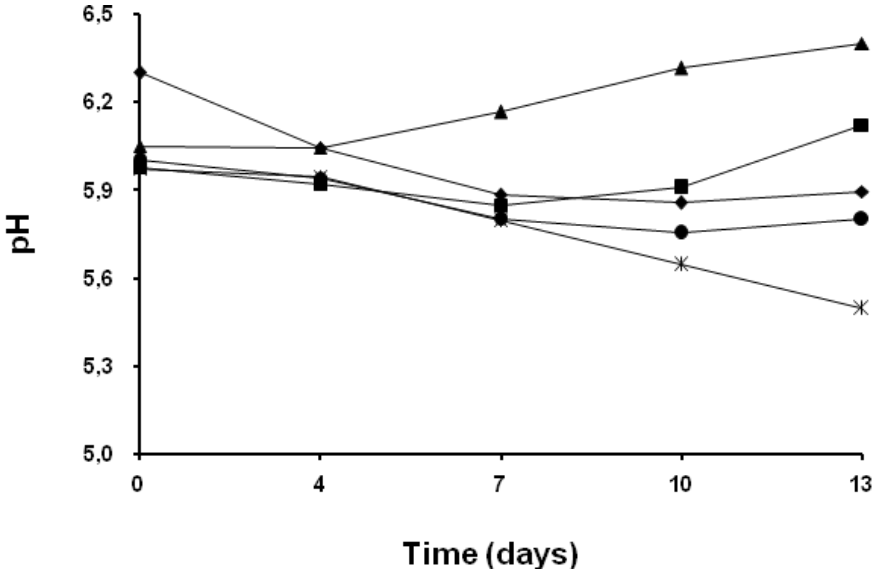

Fig. 6. Nitrogen source selection: Culture media pH. (♦) YE+P, (■) YE+CSL, (▲) YE+Asn, (●) P+CSL, and (×) YE+KNO_3

Regarding the mechanical properties of the BC (Table 2), the burst index is significantly lower with YE+Asn or YE+KNO_3, compared with the rest of the nitrogen sources. For the other three combinations, tested burst strength remains unchanged throughout the experiment, with most values ranging between 4 and 10 kPa m^2/g, clearly above those normally found on paper.

The tear strength, as in the study of carbon, shows rather low and scattered values ranging from 2 to 9 mN m^2/g; therefore, this variable lacks discriminatory capacity to guide the choice of nitrogen source (Table 2). The optical properties generally stay within the same range of values previously shown when describing the assays of the carbon sources (Table 2).

In view of the above results it was concluded that the most appropriate combinations of nitrogen sources are YE–P and YE–CSL. In the absence of significant differences between these two sources of nitrogen, the effect of ethanol was evaluated on both combinations.

Table 2. Variation of Optical and Mechanical Properties of BC Layers at Different Time Points Depending on the Nitrogen Source Used

Nitrogen Source	Time (Days)	Burst Index (kPa m^2/g)	Tear Index (mN m^2/g)	Opacity (%)	Yellowness (%)
Yeast extract + Peptone	4	4.11	5.44	16.55	12.68
	7	10.5	3.13	18.48	38.57
	10	8.91	2.69	22.16	36.91
	13	8.01	2.11	22.91	46.00
Yeast extract + Asparagine	4	b	9.34	14.76	3.08
	7	4.33	4.37	15.00	20.25
	10	3.23	3.37	14.95	30.27
	13	2.77	2.97	15.00	21.36
Peptone + Corn steep liquor	4	6.64	4.64	19.30	23.63
	7	10.1	3.66	20.32	30.91
	10	10.3	3.25	20.11	34.72
	13	9.04	3.32	18.78	34.53
Yeast extract + Corn steep liquor	4	6.83	4.64	24.48	16.48
	7	11.9	3.49	22.28	33.44
	10	10.8	2.86	25.25	37.05
	13	13.6	2.16	23.47	37.41
Yeast extract + Potassium nitrate	4	a	a	a	a
	7	a	a	a	a
	10	a	a	a	a
	13	6.32	2.52	16.04	26.73
a, BC layer not formed; b, under the sensibility limit					

Evaluation of the Effect of Ethanol

As discussed in the Introduction, the addition of 1% ethanol has been shown to increase the production of BC (Yunoki *et al*. 2004). Experiments were conducted to see whether this effect was significant for the bacterial strain and conditions. BC production was measured with preselected nitrogen sources in the presence and absence of 1% ethanol. With regard to fructose consumption (Fig. 7), the addition of ethanol to the media resulted in slightly lower figures. This effect has been reported previously by Park *et al*. (2003) and by Yunoki *et al*. (2004). Nevertheless, these authors found an increase in BC production in the presence of ethanol from the beginning of the test, whereas in this study's tests with the addition of 1% ethanol to the culture media BC production increased only in the last part of the assay in which YE+CSL was used as the nitrogen source (Fig. 8).

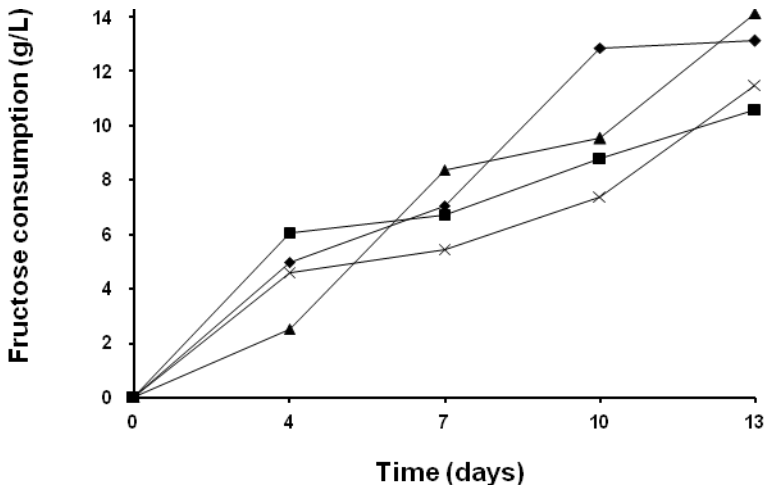

Fig. 7. Effect of ethanol: Fructose consumption. (♦) YE+P, (■) YE+P(EtOH), (▲) YE+CSL, and (×) YE+CSL(EtOH)

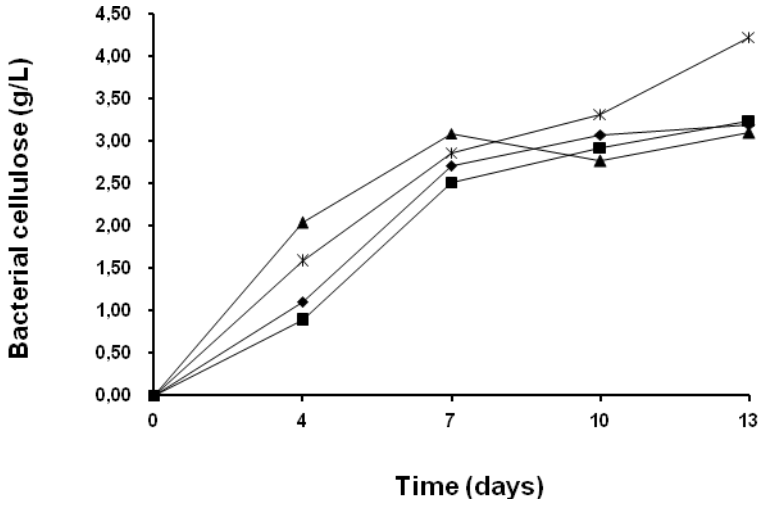

Fig. 8. Effect of ethanol: BC production. (♦) YE+P, (■) YE+P(EtOH), (▲) YE+CSL, and (×) YE+CSL(EtOH)

Both with YE+CSL and YE+P, the addition of ethanol lowered the pH (Fig. 9) more than two points on day 4. In light of what has been discussed above, the increase in the production of BC achieved with the addition of ethanol to the YE+CSL medium can only be useful if bacterial cellulose is not generated directly on the document to be restored. Using *A. xylinum* ATCC 10245, Yunoki *et al*. (2004) observed no differences in pH due to the presence of ethanol for 7 days. However, Park *et al*. (2003) used *G. hansenii* and found a decrease in pH to 3 units on day 5.

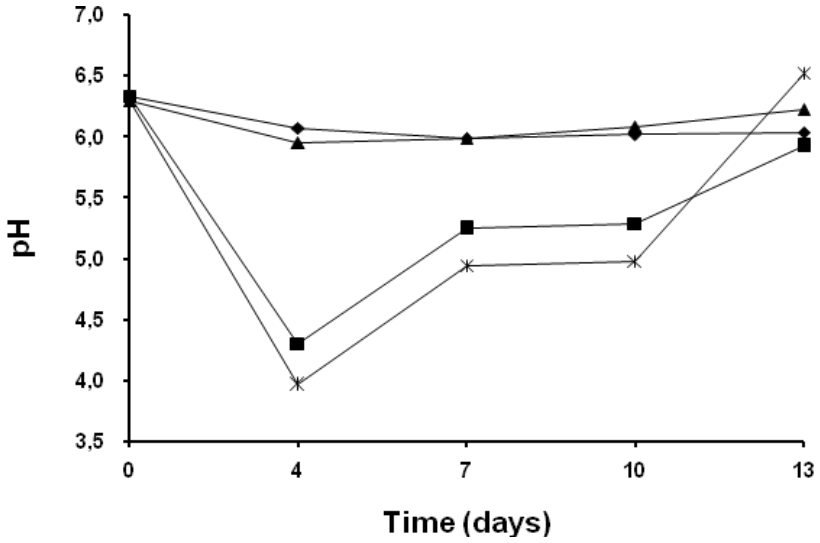

Fig. 9. Effect of ethanol: Culture media pH. (♦) YE+P, (■) YE+P(EtOH), (▲) YE+CSL, and (×) YE+CSL(EtOH)

Regarding the effect of ethanol on optical and mechanical properties (data not shown), the burst index values are similar and high in all cases, although the combination YE+CSL in the absence of ethanol provides a slightly more resistant bacterial cellulose. If the BC is going to be formed directly on the paper, YE+CSL in the absence of ethanol will be the selected culture medium as it yields a slightly more resistant bacterial cellulose and the tear strength and optical properties show no significant differences.

CONCLUSIONS

1. This study demonstrates the feasibility of using bacterial cellulose in the restoration of degraded documents.
2. Bacterial cellulose has high burst strength, acceptable tear strength, and good optical properties. In particular, cellulose produced by *G. sucrofermentans* CECT 7291 is suitable if fructose is used as the carbon source.
3. Yeast extract with corn steep is selected as the nitrogen source, without the addition of ethanol to the growth media if cellulose is to be generated directly on the paper, and with the addition of ethanol if BC is applied for reinforcement after it is formed and washed.

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