Improvement in bacterial cellulose production using *Gluconacetobacter xylinus* ATCC 10245 and characterization of the cellulose pellicles produced

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Aim

This paper deals with the improvement in bacterial cellulose (BC) production by *Gluconacetobacter xylinus* ATCC 10245 cultivated under shaking condition.

Materials and methods

The study was performed using two different approaches. First, through the addition of some thickening agents to the fermentation medium; second, by applying a two-stage fermentation process in which cells of *G. xylinus* were grown under two successive static and shaking conditions. During the first fermentation stage, the experimental microorganism was cultivated under static condition to produce thick, leather-like, white BC pellicles on which the bacterial cells were firmly adhered. However, during the second fermentation stage, these pellicles were reused to inoculate fresh, more economic medium, and incubated under shaking condition.

Results

The results showed that the addition of sodium alginate at a concentration of 0.4 g/l resulted in a BC production of 8.25 g/l compared with only 1.7 g/l obtained using the control medium without thickeners under similar shaking condition. The second method resulted in a substantial increase in the produced BC pellicle up to about 16.9 g/l. Further increase in the weight of the BC gels was obtained by adopting a repeated batch cultivation method during the second fermentation stage. After seven successive repeated batches, which extended for 56 days, the total BC mass reached 81.25 g/l. The properties of some BC pellicles were studied using thermogravimetric analyses and then compared with those of cotton linter.

Conclusion

The results showed that these two methods are very promising for BC production on a large scale.

Keywords:

bacterial cellulose, batch cultivation, *Gluconacetobacter xylinus* ATCC 10245, thermogravimetric analyses, thickening agents

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Introduction

Bacterial cellulose (BC), produced mainly by some strains of the genera *Acetobacter*, differs from plant cellulose with respect to its size, crystallinity, and purity [1]. Because of these physiochemical properties, there has been increased interest in the new fields of application of BC and in the development of new methods for its mass production in recent times [2]. *Gluconacetobacter xylinus* is a BC producer, and it is an obligate aerobic microorganism. Therefore, the conditions for its optimum growth include adequate aeration. This is why it is thought that the BC pellicle is produced at the air–liquid interface [3] and Verschuren *et al.* [4]. However, this surface pellicle is formed only on the surface of static cultures. As long as the system is kept unshaken, the disk-shaped gel was found to be suspended by its cohesion to the interior walls of the vessel [5]. Although the production process of BC under static cultivation condition was widely studied by many investigators [6,7], this process was not suitable for large scale production as the productivity was found to be highly dependent on the surface area of the medium [8,9]. However, several researchers [10–13] have found that intensive agitation and aeration drastically reduce BC synthesis, as such conditions induce the formation of a spontaneous cellulose nonproducing mutant (Cel-1), which dominates in the culture [14]. The results of a previous study performed by our team [15] showed that BC production could be optimized by cultivating *G. xylinus* ATCC 10245 in a modified corn steep liquor (CSL)/treated molasses medium for 144 h under static condition. However, the study also showed that the growth of the producing microorganism in the same fermentation medium under shaking condition resulted in very low or nonreproducible amount of BC. The present study investigates the possibility of establishing a high and stable production of BC by *G. xylinus* ATCC 10245 under shaking cultivation condition. This was achieved by assessing the effect of some factors, including the

addition of some thickening agents to the fermentation medium, as well as changing the cell cultivation conditions, on the weights, as well as the properties, of the BC pellicles produced.

Materials and methods

Plant-derived cellulose

In this study, cotton linter (CL) was used as a representative of plant-derived cellulose. It was provided by the Chemical Industries Co. (Abou-Zaable, Cairo, Egypt).

Microorganism and culture media

G. xylinus (ATCC 10245) used in this study was purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA).

Two different constitutive media were used. These media were optimized in a previous study for the static production of BC by the above mentioned microorganism [15]. Their compositions were (g/l) as follows: medium 1 (modified CSL medium): mannitol, 25.0; Na_2HPO_4, 2.70; citric acid, 1.15; and CSL, 80.0 ml. Medium 2 (modified CSL-treated sugar cane molasses medium): H_2SO_4-heat treated sugar cane molasses, 170.0; Na_2HPO_4, 2.70; citric acid, 1.15; and CSL, 80.0 ml.

Fermentation vessels and cultivation conditions

In the first experiment, the effect of the addition of different types of thickeners to the modified CSL fermentation medium on BC production was studied. The thickeners used in this study were agar, carboxy methyl cellulose, polyacrylamide, hydroxy-ethyl cellulose, and sodium alginate (SA). Each of these thickeners was individually tested at two different concentrations of 0.2 and 0.4 g/l. In this experiment, the fermentation medium was inoculated with 48-h slants of *G. xylinus* ATCC 10245, and then incubated at 30°C for either 72 or144 h under a shaking condition of 200 rpm. The shake flasks used had a nominal volume of 250 ml and a working volume of 40 ml of the previously mentioned fermentation medium. Two control modified CSL medium without thickeners were also tested for BC production under both shaking and static fermentation conditions.

In the second experiment, *G. xylinus* ATCC 10245 was cultivated under two successive stages of static and shaking conditions. Medium 1 was used for the static production of BC. This medium was, in a previous study [15], found to produce the highest amounts of BC. However, medium 2 was used for the production of BC under shaking condition because of its low cost. This media is economic and composed of byproduct of carbon and nitrogen sources (molasses and CSL, respectively) and used as an alternative to the expensive conventional ones. CSL is obtained as a byproduct of corn wet milling industry, whereas molasses solutions are obtained as a byproduct after the final crystallization stage of the sugar production industry.

In the first stage of the experiment (static stage), 48-h slants of the producing microorganism were used to inoculate six sets of 100 ml conical flask, each containing a working volume of 40 ml modified CSL medium. Each set of flasks was incubated under static condition and at 30°C for a certain incubation period, which ranged from 1 to 6 days. The second stage of the experiment (shaking stage) was performed using two different methods.

In the first method, the BC gels obtained at the end of each static incubation period were recovered under aseptic condition, washed with sterile saline solution, and then transferred to another set of 250 ml conical flasks each containing a working volume of 40 ml of modified CSL-heat treated sugar cane molasses medium. These flasks were then incubated under a shaking condition of 200 rpm and 30°C for different incubation periods ranging from 1 to 15 days. As a control, the amount of BC produced under these conditions was compared with that produced in another set of 250 ml conical flasks, each containing similar working volume of modified CSL-heat treated sugar cane molasses medium, but inoculated using 48-h slants of *G. xylinus* ATCC 10245 and incubated directly under a shaking condition of 200 rpm and 30°C for the same incubation period of 1 to 15 days.

In the second method, the BC gels obtained by the static incubation of the producing microorganism were also recovered and treated as mentioned before. They were then subjected to repeated batch cultivation by the serial addition of 40 ml batches of fresh CSL-heat treated sugar cane molasses medium every 8 days of incubation under shaking condition at 200 rpm and 30°C. At the end of each batch, the BC pellicles were gently washed with 0.9% saline solution before the addition of the fresh medium. This was performed for seven successive batches until 56 days of incubation.

Treatment of molasses

The molasses solution used in this study was supplied by the Sugar and Integrated Industries Corporation (Al-Howamdia, Egypt). It consisted of about 24.91% total solid, and 50% of this solid represented total

sugar. The crude molasses was treated according to the method of Bae and Shoda [16], in which crude molasses was diluted five-fold (w/v) with distilled water and then centrifuged at 6000 rpm for 20 min to separate solid materials. The supernatant designated as molasses solution was adjusted to pH 3.0 using 4 N H_2SO_4 and then heated at 120°C for 20 min. The obtained solution (termed as H_2SO_4-heat treated molasses) was kept overnight at room temperature and then recentrifuged.

Determination of bacterial cellulose yield

At the end of each fermentation period, the BC gels formed on the surface of the fermentation media were carefully recovered, washed, and immersed overnight in 1 N NaOH solution at room temperature to remove bacterial cells and excess media components. The BC gels were then thoroughly washed with distilled water until the pH of the washing water reached normal ranges (Toda *et al.*, 1997) [17]. The BC gels were then immersed in 96% alcohol, followed by immersion in diethyl ether solution for few hours. Finally, the BC gels were carefully dried by gentle heating (about 40°C) and then weighed to compare the BC production ability of the producing microorganism under different fermentation conditions.

Characterization of bacterial cellulose

Thermal analysis

Thermogravimetric analyses (TG and DTG) of the examined cellulose samples were carried out using Perkin Elmer thermogravimetric analyzer (USA) (TGA 7). The analyses were performed at a heating rate of 10°C/min and a flow rate of 50 ml/min, under nonisothermal conditions and in the presence of nitrogen [18].

TG-curve analysis

Kinetic studies based on the weight loss data were obtained with TG curve analysis. The activation energy has been evaluated by applying the Coat and Redfern method of analysis [19]. For pseudohomogeneous kinetics, the irreversible rate of conversion of the weight fraction of reactant was expressed by the following equation:

$$\frac{d\alpha}{dt} = k\,(1-\alpha)^n, \quad (1)$$

where, α is the fraction of material decomposed at time t, k is the specific rate constant, and n is the order of reaction. The temperature dependence of k is expressed by the Arrhenius equation:

$$k = Ae^{-E_a/RT}, \quad (2)$$

where A is the frequency factor (s^{-1}) and T is the absolute temperature.

For linear heating rate, a is as follows (degree/min[1]):

$$a = \frac{dT}{dt}. \quad (3)$$

For calculating the activation energy, E_a, of thermal decomposition when n=1, Eq. (4) was used.

$$\log\left[-\log\frac{1-\alpha}{T^2}\right] = \log\frac{AR}{aE_a}\left[1-\frac{2RT}{E_a}\right] - \frac{E_a}{2.3RT}. \quad (4)$$

When n[1] ≠ 1, Eq. (5) was used;

$$\log\left[\frac{1-(1-\alpha)^{1-n}}{T^2(1-n)}\right] = \log\frac{AR}{aE_a}\left[1-\frac{2RT}{E_a}\right] - \frac{E_a}{2.3RT}. \quad (5)$$

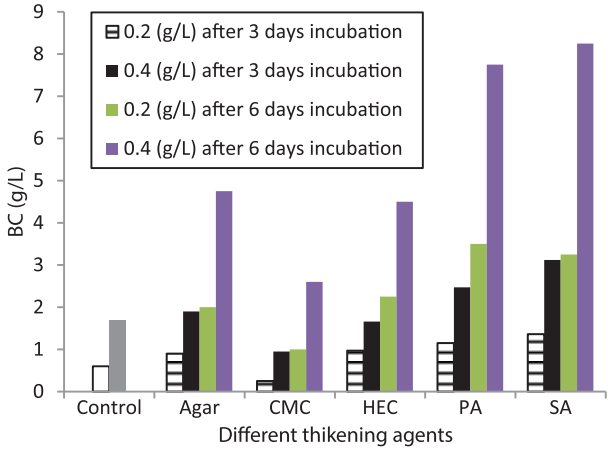
Plotting the left-hand-side value of the equation {i.e. log $[1-(1-a)^{1-n}/T^2(1-n)]$} against $1/T$ using various values of 'n' should give a straight line with the most appropriate value of 'n' [20]. The least square method was applied for the equation, using values of 'n' ranging from 0.0 to 3.0 in increments of 0.5. The correlation coefficient (r) and the SE were calculated for each value of 'n'. The 'n' value, which corresponds to the maximum r and minimum SE, is the order of the degradation process. The activation energies and frequency factors were calculated from the slope and intercept, respectively, of the Coat–Redfern equation with the most appropriate value of 'n'.

Results and discussion

Effect of the addition of different thickeners, for two different incubation periods, on the improvement of BC production under shaking cultivation conditions

Figure 1 illustrates the effect of the addition of different thickeners at two different concentrations of 0.2 and 0.4 g/l on BC production by the microorganism

Figure 1



Effect of different thickening agents on bacterial cellulose (BC) production.

cultivated with shaking for two different incubation periods of 72 and 144 h.

The results presented in Fig. 1 show that, after 144 h of incubation, the maximum yield of BC of 8.25 g/l was obtained when SA was added to the fermentation medium at a concentration of 0.4 g/l, and that lower amounts of BC of 7.75 g/l were obtained when polyacrylamide gel was added at the same concentration.

However, the addition of similar concentrations of agar, hydroxy-ethyl cellulose, or carboxy methyl cellulose for the same incubation period, resulted in lower BC amounts that ranged from 2.25 to 4.75 g/l. In contrast, the BC titers recorded, either in the fermentation medium to which the tested thickeners were added at a lower concentration of 0.2 g/l or for a shorter incubation period of 72 h or even in the control medium without any thickeners added, were much lower.

In contrast, the results showed that there were no significant differences in the enhanced amount of BC (8.1–8.3 g/l) produced as the concentration of SA was increased from 0.4 to 1.0 g/l. As a result, SA was selected as the optimal additive for increasing BC production by *G. xylinus* ATCC 10245, cultivated under shaking condition for 144 h.

The obtained results are in accordance with those of several investigators [21,22], who proved that BC production is enhanced if the viscosity of the culture, incubated under shaking condition, was maintained at high levels. This was achieved by the addition of some thickeners, such as agar and polyacrylamide, to the fermentation medium.

According to Bae *et al.* [22], the addition of such substances to the fermentation medium resulted in the production of smaller BC pellets. This is advantageous with respect to the transfer of nutrients and oxygen to the bacterial cells located inside as well as those located on the surface of the BC pellets matrix.

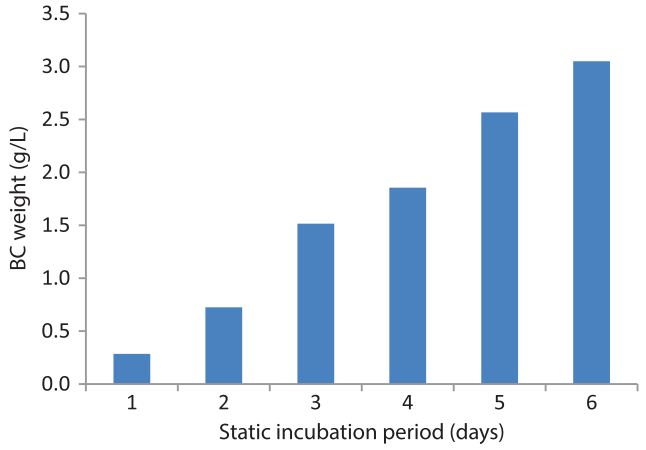
Effect of the cultivation of *Gluconacetobacter xylinus* (ATCC 10245) under two successive stages of static and shaking conditions

The results of the first stage of the experiment (static cultivation of the producing microorganism) illustrated in Fig. 2 show that BC yield increased from 0.285 to 3.05 g/l, as a result of increasing the static incubation period from 1 to 6 days. Moreover, the results of the second fermentation stage (shaking cultivation of the statically obtained BC pellicles) presented in Fig. 3 show that, when cultivated under shaking condition, the weight of these BC pellicles was found to be substantially increasing as the age of the inoculums increased from 1 to 6 days of static cultivation.

Moreover, the study proved that the cultivation of these statically produced BC pellicles under shaking cultivation condition (first method in the second stage of the experiment) resulted in much higher and reproducible BC titers than that obtained when the producing microorganism was directly incubated under shaking conditions. The data are not shown due to triplicate variability.

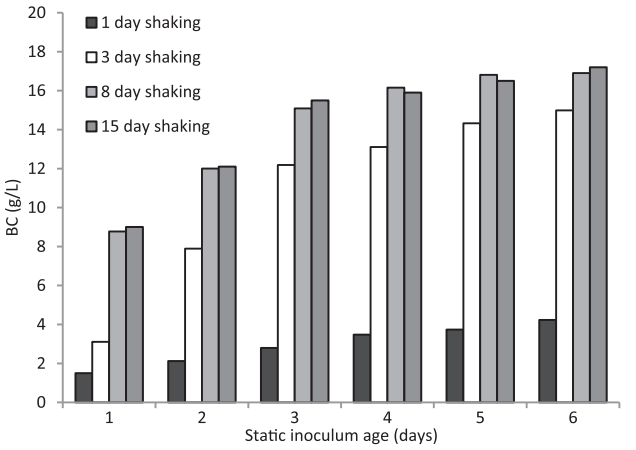
In contrast, the results proved that, generally, the incubation of the BC gels, (resulting from the static cultivation of *G. xylinus* cells) under shaking conditions resulted in a continuously increasing yield of BC until 8 days of incubation period. A maximum amount of BC of about 16.9 g/l was reached when the producing microorganism was incubated for 6 days under static condition, followed by the incubation of the produced

Figure 2



Effect of different static incubation period on bacterial cellulose (BC) production.

Figure 3

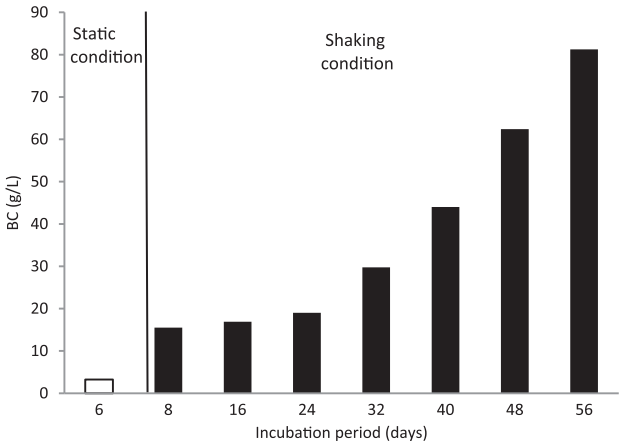


Effect of different static inoculum ages on bacterial cellulose (BC) production under shaking conditions for different incubation periods.

BC pellicle for 8 days under shaking condition. However, when the shaking incubation period was extended until 15 days, no increase in BC production was noticed and a plateau was formed. This was because the amount of BC produced gradually increased in the conical flasks and therefore the fermentation medium became insufficient for sustaining more BC production.

Finally, the second fermentation stage was performed using a second method, which was the repeated batch cultivation method. This involved the shaking cultivation of the BC pellicles, originally produced by the static cultivation of *G. xylinus* (ATCC 10245) for 6 consecutive days, using seven successive batches of fresh modified CSL-treated sugarcane molasses medium. The results presented in Fig. 4 show a gradual increase in the weight of the BC pellicles as the incubation time increased. During the first batch incubation under shaking condition, the recorded rate of BC productivity was 1.8 g/l/day compared with 0.5 g/l/day productivity of the same microorganism under static cultivation condition. Consequently, 15.5 g/l of BC resulted after only 8 days of the shaking incubation of a 3.25 g/l BC pellicle, obtained by the static incubation of the test microorganism for 6 days (about 4.8-fold increase in BC weight). This rate then gradually decreased as the experiment proceeded, reaching 0.8 g/l/day during the third batch of incubation. However, the productivity of the producing microorganism then gradually increased and reached 1.4 g/l/day at the end of the experiment. Therefore, after 56 days of shaking incubation at 200 rpm and 30°C, the weight of BC pellicles had increased from 3.25 to 81.25 g/l as a result of their subjection to seven successive batch additions of 40 ml batches of economic medium. Each batch lasted for 8 days of incubation. This result represents a 25-fold increase in the weight of the produced BC gel. The experiment was terminated after the above mentioned incubation period of 56 days as no sign of regression was observed; therefore, the experiment was expected to last as long as the volume of the fermentation vessel was adequate for such increase in the weight and consequently in the volume of the produced BC mass.

Figure 4



Repeated batch production of bacterial cellulose (BC) under shaking cultivation condition.

Comparative studies of the properties of bacterial cellulose and plant-derived cellulose

To clarify the difference between some properties of the BC that resulted from the different cultivation techniques, thermal analysis of some selected BC pellicles was carried out for comparison with CL, which is a natural-based cellulose. This was performed by subjecting the BC and CL samples to thermogravimetric analyses under nonisothermal conditions.

Three cellulose samples were tested. They represented BC pellicles obtained by the cultivation of the producing microorganism cells under three different cultivation conditions:

Sample 1: Comprised BC pellicles obtained by the static cultivation of the producing microorganism for 6 consecutive days.

Sample 2: Comprised BC pellicles obtained by the cultivation of the producing microorganism statically for 6 days and then under shaking condition for an additional fermentation period of 8 days.

Sample 3: Comprised BC pellicles obtained by the static cultivation of the producing microorganism statically for 6 days and then under shaking condition for 56 days' incubation time using repeated batch cultivation every 8 days.

The thermal stability of each of these samples, as well as that of CL, was estimated and compared taking into account the value of the initial decomposition temperature, maximum weight loss temperature, the correlation coefficient (r), standard error estimation (Se), and the activation energy associated with each degradation stage as a function of order 'n' (Table 1). The maximum weight loss temperature was the temperature at which the derivative weight of the thermogravimetric analyses curve reached a maximum.

Table 1 clearly elucidates that the thermal degradation of CL cellulose took place through two main degradation stages, which followed the evolution of absorbed moisture. The first main degradation stage resulted from the formation of carbonyl and carboxyl groups, evolution of carbon dioxide and carbon

Table 1 Thermal degradation measurements of bacterial cellulose samples produced using different cultivation techniques in comparison with those of plant-derived cellulose

BC origin	Main degradation stage	Temperature range (°C)	Maximum weight loss temperature (°C)	Order *n*	*r*	Se	E_a (KJ/mole)
CL	1st	301.1–370.7	342.2	0.5	0.9939	0.0513	245.71
	2nd	447.8–515.2	501.2	1.5	0.9946	0.0667	363.55$\sum E_a$=609.26
Sample 1	1st	249.7–398.9	350.9	1.0	0.9986	0.0267	96.11
		398.9–499.8	496.8	1.0	0.9897	0.0750	167.9
	2nd	499.8–561.2	525.6	2.5	0.9895	0.1340	794.11$\sum E_a$=1058.12
Sample 2	1st	50–174.8	71.06	–	–	–	–
		196.9–331.65	257.23	1.5	0.9908	0.0931	108.461
	2nd	503.76–614.06	557.96	1.5	0.99608	0.0576	289.032$\sum E_a$=397.49
Sample 3	1st	50–167	66.05	–	–	–	–
		193.06–324.7	253.86	1.0	0.99815	0.0595	70.9624
	2nd	502.76–588.71	573.90	0.5	0.9874	0.0894	302.082$\sum E_a$=373.044

BC, bacterial cellulose; CL, cotton linter.

monoxide, and formation of carbonaceous char. This stage is called the volatilization stage. The second main degradation stage was related to the rapid volatilization and oxidation of char, accompanied by the formation of carbonaceous residue. This stage is called the carbonaceous stage [19].

Thermal degradation of BC took place over more than two degradation stages, in addition to the stage corresponding to the sorbet moisture. This additional stage proves that BC was formed in two different degrees of crystallization. Therefore, the first two stages were regarded as volatilization stages and consequently considered as the first main degradation stage, whereas the following degradation stage was regarded as the second main degradation stage (carbonaceous stage).

However, it was noticed that, although the volatilization (first main degradation) stage of Cl samples started at a higher temperature (of about 300°C) than that of the BC samples (which varied from 50 to about 250°C), the carbonaceous (second main degradation) stage began at a lower temperature of less than 450°C compared with around 500°C for BC samples.

Moreover, the table reveals that the thermal degradation property of the sample 1 BC (grown under static condition) differs from that of samples 2 and 3 BC (grown using the two stages fermentation process) as the onset of the first main degradation stages of sample 1 BC was recorded at higher temperatures of about 250°C compared with samples 2 and 3 BC, for which the volatilization stage started at only 50°C. However, that of the second degradation stage was more or less equal in all tested BC samples (around 500°C).

In contrast, the results show that the major loss in the weight of samples 2 and 3 BC took place over a wider range compared with CL and sample 1 BC, revealing a higher resistivity for thermal degradation as a result of their production in the previously described two phases fermentation process.

Furthermore, the calculated total energy of activation, detected for all degradation stages of each sample, was much higher for sample 1 BC than for the other tested BC samples, proving that the subjection of the BC pellicles, obtained as a result of the static cultivation of the producing cells, to shaking growth conditions resulted in a significant variation in their chemical properties.

Conclusion

First of all, the results of the addition of SA to the fermentation medium at the final concentration of 0.4 g/l resulted in an improved BC production of 8 g/l under shaking cultivation condition.

In contrast, the two stages fermentation process (including static cultivation, followed by repeated batch shaking cultivation) was performed to evaluate the possibility of increasing the amount of BC production by repeated batch shaking cultivation of the statically obtained BC pellicles. The experiment relied on the ability of the *G. xylinus* cells, which are firmly adhered on the surface of the BC pellicles, to sustain a constant BC increasing production ability, as long as they were continuously provided with adequate amounts of the production medium. The results proved that the growth and rate of BC production by the tested bacterial cells were more or less maintained by the successive addition of the fermentation medium as mentioned before. This lead to the conclusion that the production of BC by *G. xylinus* cells immobilized by their adherence on the surface of the statically produced BC pellicles was a very promising method for BC continuous production on a large scale. Furthermore, the result of the

thermogravimetric analyses showed that the resulting BC pellicles had improved thermal properties as well as chemical activity.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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