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Physicochemical properties of bacterial cellulose obtained from different Kombucha fermentation conditions

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Abstract

The production of bacterial cellulose has been limited due to its high cost and low productivity. Alternative low-cost sources of this biopolymer of high purity and biocompatibility are needed in order to benefit from its enormous potential. Kombucha tea is a trend functional beverage whose production is growing exponentially worldwide, and the bacteria present in this fermented beverage belonging to the genus *Komagataeibacter* are capable of producing a crystalline biofilm with interesting properties. Obtaining bacterial cellulose from Kombucha tea has already been studied, however several fermentation conditions are being optimized in order to scale-up its production. In this study, we characterized the bacterial cellulose produced from three different Kombucha fermentation conditions. The scanning electron microscopy images revealed the crystalline structure of the biofilms. The energy-dispersive x-ray analysis exhibited the chemical composition of the crystals. The thermogravimetric analysis showed a rate of degradation between 490 and 560°C and the differential scanning calorimetry confirmed the presence of crystalline and amorphous regions in the bacterial cellulose samples. The results suggested that crystalline cellulose could be obtained by varying the fermentation conditions of Kombucha tea.

KEYWORDS

bacterial cellulose, fermentation conditions, Kombucha, SEM, TGA

1 INTRODUCTION

Nowadays, bacterial cellulose (BC) is a promissory material for the development of biotechnological devices in many different fields, especially in the medical one, where it has been already used as wound dressing to heal lower extremity ulcers and it was observed that it shortened the healing time as compared to standard care.[1] BC nanocomposites were also evaluated for its use in bone and cartilage tissue engineering, optical materials development, membranes for separation processes and energy storage devices.[2] Recently, Zahan et al.[3] evaluated its application as a biodegradable and antimicrobial packaging and observed 100% of degradation after 7 days in the soil. However, the availability and production costs of this biofilm still need to be improved. Currently, its production from different microorganisms is being optimized with the aim of reducing the production costs, and scale up optimization is a technological prerequisite to be adopted at large scale.[4] During the formation process of the cellulose pellicle various carbon compounds of the nutrition medium are utilized by the *Komagataeibacter*

sp., which has been reported as the dominant genre in Kombucha consortiums.[5] These compounds are formerly polymerized into single, linear ß-1,4-glucan chains and finally secreted outside the cells through a linear of pores located on their outer membrane.[6-8] These chains with a ribbon-like structure will self-assembly into fibrils creating a macrofibril network that may vary depending on the used strains, culture time and chemical additives present in the culture media.[9] BC has distinctive advantages over traditional sources: no delignification is required following harvest,[10] its physical properties such as crystallinity, hydrophilicity, and degree of polymerization are superior than those from the plant-derived cellulose,[11] and it can be produced from a wide variety of substrates.[12] One particular source for BC production is Kombucha tea, where it is produced as a floating biofilm associated with a consortium of bacteria and yeasts, and according to Nguyen et al.[13] it may also have different characteristics than those from typical sources. However, Kombucha tea elaboration process is facing a standardization phase in order to do its scaling-up for industrial production[14] and several parameters which could modify the BC yield have already being studied.[15] Such as, temperature,[16] fermentation time,[17] depth, and surface area,[18] between others. Moreover, although yield is important, further research is needed in order to study the impact of the processing over the physicochemical properties of the BC. The aim of this study was to compare different fermentation parameters of Kombucha tea and their influence over the characteristics of the final biofilm.

2 MATERIALS AND METHODS

2.1 Culture conditions and inoculum preparation

2.1.1 Starter inoculum

The tea fungus was purchased from the website www.jemange-vivant.com and maintained in sugared black tea according to an optimized protocol in the laboratory.

2.1.2 Preparation of Kombucha teas

Ten gram of Ceylon black tea and 70 g of sucrose were added to 1 L of boiling water and allowed to infuse for 15 minutes, the tea was then removed and the infusion was left to cool. Once the temperature was around 25°C the tea was inoculated with 20 g of the starter inoculum and 20 mL of previously fermented medium. Finally, the beakers were covered with cheesecloth for allowing a proper aeration, and were then incubated at 25°C for 15 days.

Three different cultivation methods were tested:

- Tea broth in a static fermentation (control)
- Tea broth in a static fermentation plus 2 g/L of yeast extract
- Tea broth with magnetic stirring at 100 rpm

2.2 BC samples preparation

The BC biofilms were removed from Kombucha fermented tea and directly dried at 60°C during 24 to 36 hours without any specific cleaning procedures. Once dried they were cut in small pieces according to each analytical technology.

2.3 Scanning electron microscopy equipped with energy-dispersive x-ray images

Then, 1 cm^2 of the sample was plunged into a 4% glutaraldehyde solution during 2 hours in order to preserve the biological material. Then it was washed during 5 minutes into different mixes of Acetone/water (70%-30%) and Acetone/HMDS (hexamethyldisilazane) (50%-50%) to be dehydrated. Finally, a metallic coating was performed under vacuum conditions to provide the sample with a conductive layer during analysis. Images were performed with a JEOL-JSM-6700F instrument. Magnification range used on scanning electron microscopy (SEM) images was X100 - X25000. For EDX analysis, biofilm sections were fixed on holders and 6 nm sputter-coated with carbon by means of a MED 020 sputter device (Bal-Tec). EDX analyses were performed at 5 and 10 kV.

2.4 Thermogravimetric and derivative thermogravimetric analysis

Both scans were performed under air condition by a TGA Q600 analyzer from TA Instruments. About 8 mg were introduced inside an alumina crucible for each test. Temperatures were tested in the range of 25 to 1000°C. Thermogravimetric (TG) and derivative thermogravimetric (DTG) analysis were used to approach the thermal properties of the Kombucha membranes as onset temperature degradation (T_{on}) or temperature of maximum rate of degradation (T_d).

2.5 Differential scanning calorimetry scans

The analysis were performed with a Q2000 instrument from TA Instruments. The Q2000 differential scanning calorimetry (DSC) is a heat-flux DSC with Modulated DSC capability. Five milli gram of sample were placed in an aluminum DSC support with a standard aluminum lid. Samples were tested under an air flow of 50 mL/min. The rank of temperature used was 10°C/min from −50 to 400°C. The DSC analysis was used to study the thermal properties of the Kombucha biofilms as glass transition temperature (T_g), crystalline phase transition temperature (T_c), and denaturation temperatures.

3 RESULTS AND DISCUSSION

3.1 SEM analysis

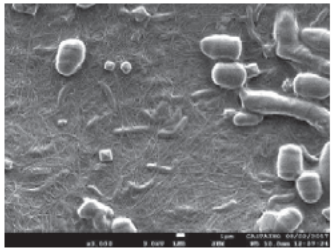
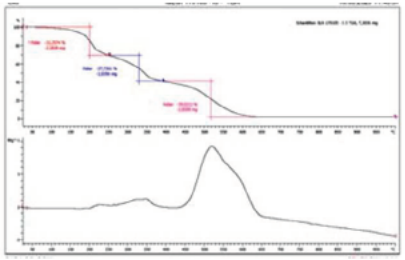
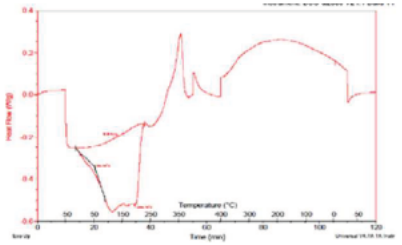
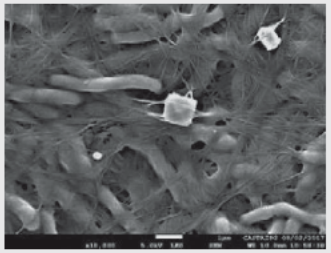
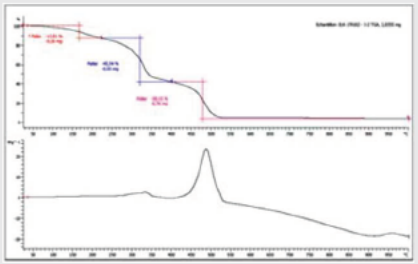
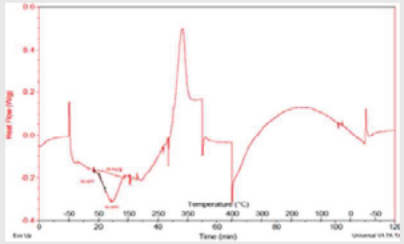
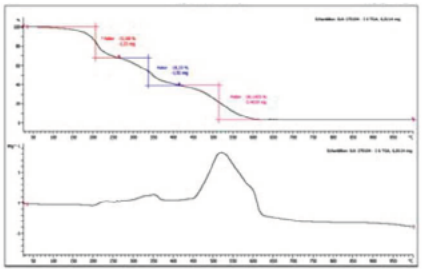
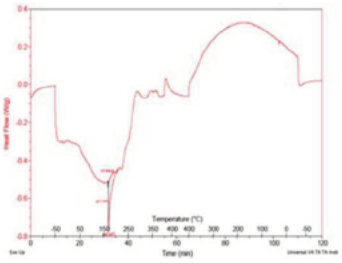
According to the American Food and Drug Administration (FDA), Kombucha tea must be fermented between 7 and 10 days in order to be consumed, as the concentration of organic acids might reach an unsafe level for human consumption. In this study, we fermented for 15 days in order to find a compromise between the beverage sensorial acceptance and its biofilm production. This biofilm acts as a nutrient source by containing several proteins, extra cellular enzymes, nucleic acids, lysed cells, and so on.[19] It is composed of an entanglement of microfibrils of cellulose which conforms the pellicle present some crystals that are formed due to a highly ordered fibrillary cellulose orientation (about 70%-80%). These components represent an important energy source during fermentation; however, they are considered organic impurities when studying the biofilm structure, as they remained attached to the surface of the gel as seen in Table 1. SEM offered important visual information on the morphology of the biofilm samples. One-micron resolution scans showed entanglements of microfibrils with a diameter lower than 100 nm. Ovoid and stick forms were microorganisms from 1 to 3 μm, some of them were present on the surface of the sample and others were caught into by the fibrils and forced to remain into the matrix. It can be seen that the higher microbial population was found in the magnetic stirring condition, probably due to the agitation, which deliver most of the cells into suspension. A higher presence of microbial cells can prevent the contact between the fibrils within the network, reducing the number of hydrogen bonds and thus the strength of the biofilm.[20] This theory was observed in the studied samples, as the microfibrils seemed to constitute a more organized network in the case of the static condition. Our obtained biofilms present a denser matrix than those observed by Lee et al.[9] where their biofilm was characterized after 7 days. Crystalline forms of 0.5 to 1 μm were present on the SEM images of all the different cellulose biofilms and BC is known for having a high crystallinity of around 60% to 80%.[13] Despite this property is highly desired as it tends to improve the mechanical properties of the polymer,[21,22] worked with a mechanochemical reactor in order to decrease the crystallinity of the cellulose and create a functional additive to enhance melt processability of a water-soluble synthetic polymer.

3.2 EDX analysis

EDX analysis applied on the matrix showed two major atoms represented in the samples (Figure 1): Carbon (50%-62%) and Oxygen (38%-50%). These two major atoms constituted the structure of the fibrils. These results were consistent with the cellulose structure because cellulose formula $(C_6H_{10}O_5)_n$ presents 55% of C and 45% of O if H atom are neglected in the calculation. Hydrogen atoms are not considered because these are not measurable in EDX analysis because of its extremely small photoelectron cross-section. However, traces of nitrogen were observed in some spectra, which could probably come from contaminants of the Kombucha biofilms as amino-acids or/and proteins from entangled microorganisms. Jayabalan et al.[23] performed a proximate analysis of the biochemical constituents of a Kombucha biofilm and found Oxygen and Carbon as the major compounds. Nevertheless, they detected also other compounds such as, Na, K, Mg, Zn, and Ca, whose concentrations increased progressively during fermentation.

Figure 2 shows the comparison between the EDX analysis of a crystal (spectrum 5) and of the matrix (spectrum 6). Spectrum 5 showed a Calcium peak which was not present on spectrum 6 and showed also a higher peak of Oxygen. The detected elements in the spectrum 5 suggest that it could be formed of $Ca(OH)_2$ crystals. Podolich et al.[24] carried out similar analysis and detected calcium and potassium in their Kombucha biofilms. However, these elements were not detected in the characterization done by Mohite and Patil[25] who just detected carbon and oxygen in 54.06% and 45.94%, respectively in a biofilm produced by *Gluconacetobacter hansenii*. The differences observed in the chemical composition of the bacterial biofilms may be due to the initial microbial pop-

TABLE 1 Comparison of the physicochemical properties of the biofilms obtained with each fermentation condition [Color table can be viewed at wileyonlinelibrary.com]

Sample	SEM	TGA/DTG	DSC
Control			
Yeast extract			
Magnetic stirring			

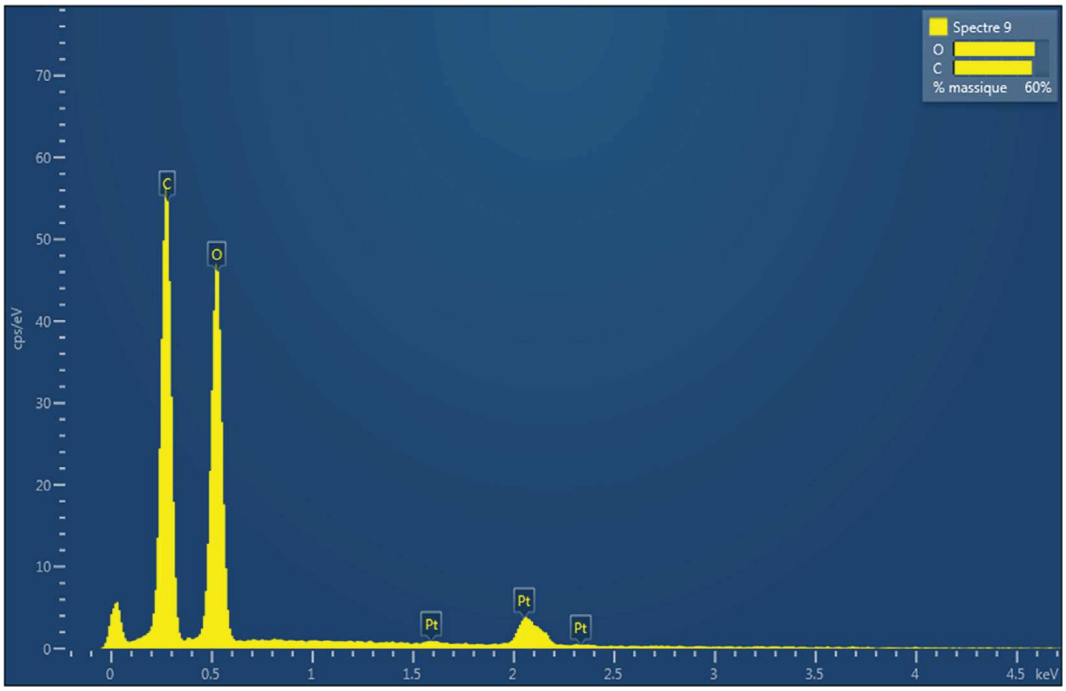


FIGURE 1 Energy dispersive x ray spectrum of the bacterial cellulose matrix [Color figure can be viewed at wileyonlinelibrary.com]

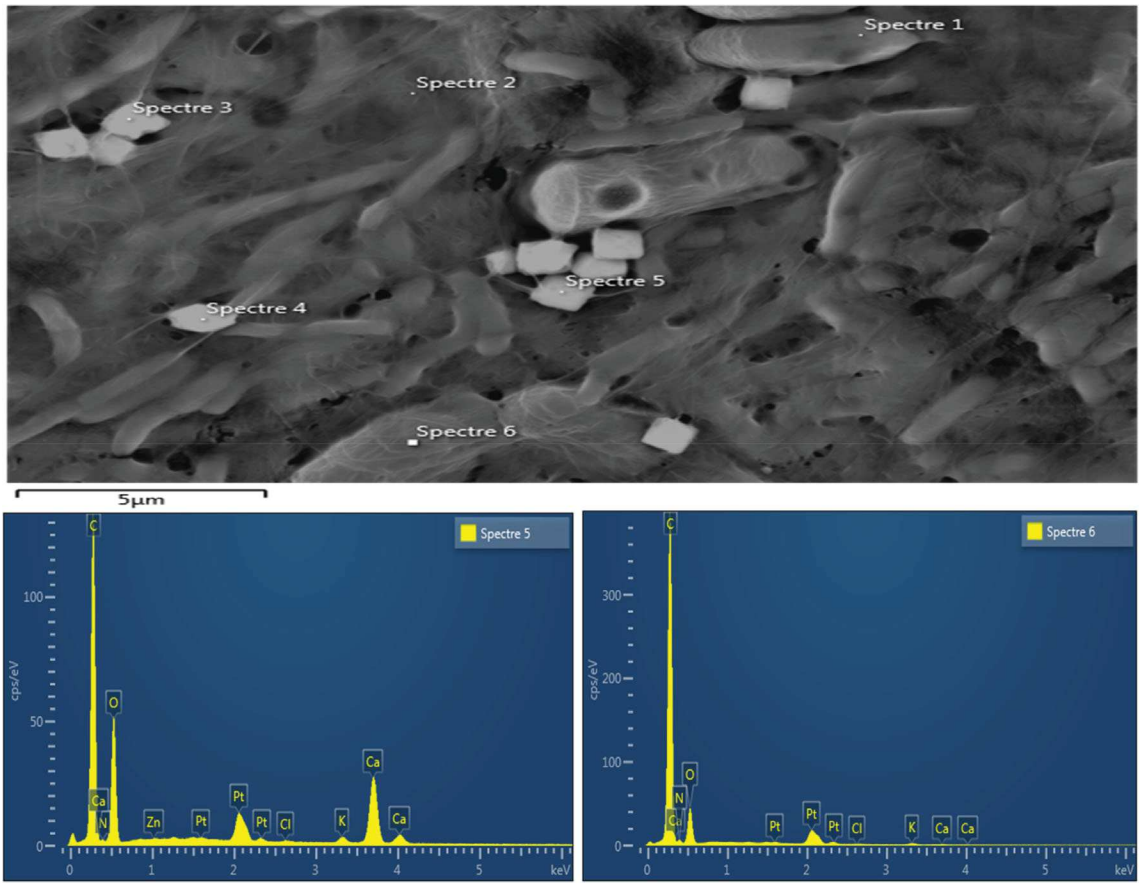


FIGURE 2 Corresponding energy dispersive x ray spectra of the bacterial cellulose matrix and crystals [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Thermogravimetric results from the different biofilm samples

Samples	First weight loss (% of total mass)	Second weight loss (% of total mass)	Third weight loss (% of total mass)	T_{on} (°C)	First T_d (°C)	Second T_d (°C)
Control	28.52 ± 3.8	27.85 ± 0.1	41.26 ± 3.1	242.5 ± 3.5	325 ± 7.0	540 ± 28.2
Yeast extract	12.43 ± 0.5	45.17 ± 0.5	37.94 ± 0.2	220 ± 0.0	320 ± 0.0	485 ± 7.0
Magnetic stirring	32.3 ± 0.5	28.68 ± 0.6	37.22 ± 1.5	260 ± 0.0	337.5 ± 3.5	522.5 ± 10.6

ulation used for its production and could lead to different final properties.

However, the fermentation conditions did not seem to affect the structure and composition of the fibrils nor their respective mass yields (~95%). In the case of the pellicles used for this study, a shotgun metagenomics analysis was done,[5] and the dominant bacterium and yeast were identified as being *Komagataeibacter rhaeticus* and *Brettanomyces bruxellensis*, respectively. Where almost 80% of the microbes present belong to the *Acetobacteraceae* gram-negative family.

3.3 TG and DTG analysis

TG and DTG analysis were performed under air conditions. All TG analysis showed three distinguished weight losses (first: 25-200°C, second: 250-400°C and third: 400-600°C) and DTG analysis presented a two step-process to degrade Kombucha biofilm elements (Table 2). Kombucha biofilm samples from the magnetic stirring condition showed the highest first weight loss of 32.3% instead of 28.52% or 12.43% for the other conditions. This difference may be explained by a higher presence of impurities, which could be normal either because of the higher yeast concentration or by the fact that magnetic stirring promoted a greater homogeneity and a higher concentration of the bacteria and yeasts in the medium that could have ended trapped in the biofilm matrix. This noncellulosic molecules inside the cellulosic matrix could also disturb the crystalline zones of the biofilm structure by releasing free hydroxyl functional groups on glycosidic units. These free hydroxyl groups could bind water molecules with hydrogen bonds and retain them into the matrix increasing its water content. This first weight loss was generally higher in comparison with values found by Machado et al.[26] who obtained a weight loss from water vaporization of ~5%. However, the samples showed a similar profile to the one observed by Mohammadkazemi et al.[12], were the loss was observed at 100°C and it was due to the loss of water. They worked with five different carbon sources and observed that the moisture content was the same for all the BC samples. Onset degradation temperature (T_{on}) and first maximum rate of degradation temperature (first T_d) were lower in the yeast extract sample (T_{on}: 220°C/first T_d: 320°C) than in the control and stirred samples (T_{on}: 240-260°C/first T_d: 320-340°C, respectively). These degradation temperatures concerned first noncellulosic compounds degradation, then cellulose depolymerisation and the decomposition of glycosidic units into gases. According to the literature, the decrease in the thermal stability can be related to a higher crystallinity index.[27] The second maximum rate of degradation temperature occurred between 490 and 560°C, which was higher than the one obtained by Gea et al.[20] of around 360°C from cellulose produced by *Acetobacter xylinum*. This could suggest that BC obtained from the Kombucha consortium seems to have more thermal stability than the one produced from only one bacterial species. However, it was lower than the one from Amarasekara et al.[28], who worked with purified cellulose and obtained its second degradation at 600°C, showing that a purification step may improve cellulose stability. The broad range of temperature values observed in the TG analysis were probably linked to the different natures, sizes and concentrations of the char residues. In order to confirm the presence of these carbonaceous materials TG scans under nitrogen atmosphere should be performed.

3.4 DSC analysis

The DSC curves indicated the energy consumed and released by Kombucha biofilms under temperature variations (Table 1). The DSC thermograms exhibited rough results because of the high contamination of the samples. However, the yeast extract sample presented a more accurate curve with isolated peaks. This confirmed the higher purity of the sample compared to the control and the stirred one as shown before. A first endothermic peak around 100°C was observed. The endothermic reactions occurred at this temperature were mainly attributed to the removal of the water and other volatile noncellulosic

compounds absorbed inside the cellulose matrix.[24] In this first peak, glass transition probably occurred but its transition temperature (T_g) was difficult to be determined. A temperature decrease after water removal might be a solution to approach T_g. The melting of the crystalline phase of cellulose is obtained at a temperature of 80 to 140°C,[25] which corresponds to the endothermic peaks observed in the case of the control and yeast extract samples (Table 1). Nevertheless, nearly no phase transition was observed in the case of the magnetic stirring condition, probably meaning that this polymer showed an amorphous structure. A second endothermic peak in the range of 200 to 250°C corresponded to the noncellulosic contaminants and cellulose degradation. A first exothermic peak around 350°C indicated exothermic chemical reactions in the oxidization of cellulose. The second exothermic peak around 375°C indicated exothermic chemical reactions in the oxidization of char residues. The last part of the curves from 400 to −50°C did not show interesting elements because all volatile compounds were already removed, and samples were completely degraded. These results completed TG and DTG results and highlight the high thermal properties of Kombucha cellulosic biofilms.

4 CONCLUSIONS

The biofilms presented a good thermal stability obtaining their maximum degradation rate around 560°C. The EDX scans revealed cellulose as the main constituent of Kombucha biofilms. An organized and tight entanglement of 80 nm macrofibrils with a crystalline morphology was observed in the SEM images of the three samples. The different fermentation conditions did not change the composition of the cellulose network. This could allow obtaining Kombucha teas according to the industrial needs and producing a crystalline cellulose biofilm at the same time. This biofilm exhibited high mechanical and thermal properties as shown on the different TG scans. Kombucha tea fermentation proved to be a promising alternative for the production of this renewable natural resource.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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