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Optimizing the Production Process of Bacterial Nanocellulose: Impact on Growth and Bioactive Compounds

Running head: Optimizing Bacterial Cellulose Production and Characterisation

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SUMMARY

Research background. The field of research on bacterial cellulose production has been growing rapidly in recent years, with the potential for its use in various applications, such as in the medical and food industries. Previous studies have focused on optimizing the production process through various methods, such as using different carbon sources and manipulating environmental conditions. However, further research is still needed to optimise the production process and understand the underlying mechanisms of bacterial cellulose synthesis.

Experimental approach. We have used Plackett-Burman and Box-Behnken experimental designs to analyse various factors impact on bacterial cellulose production. The optimized medium was analysed for fermentation kinetics, and the cellulose produced was characterised. This approach was used because it allows for the identification of significant

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factors impacting bacterial cellulose growth, the optimisation of the culture medium, and the characterisation of the produced cellulose.

Results and conclusions. The results indicated that higher sucrose concentrations, higher kombucha levels, and lower symbiotic culture of bacteria and yeast size were the most significant factors for improving bacterial cellulose production, while the others had no relevant impact. The optimized medium showed an increase in the concentration of total phenolic compounds and total flavonoids, as well as relevant levels of antioxidant activity. The pure bacterial cellulose produced showed high water absorption capacity, in addition to high crystallinity and thermal stability.

Novelty and scientific contribution. The study makes a significant scientific contribution by optimizing the culture medium to produce bacterial cellulose in a more productive and efficient way. The optimized medium can be used for producing a kombucha-type drink containing a high content of bioactive compounds and the production of bacterial cellulose with high crystallinity and thermal stability. Additionally, the study highlights the potential of bacterial cellulose as a highly water-absorbing material with applications in areas such as packaging and biomedical engineering.

Keywords: bacterial cellulose; fermentation; statistical optimisation; phenolics; antioxidant activity; kombucha

INTRODUCTION

Kombucha is a fermented drink that originated in Northeast China, typically made by fermenting *Camellia sinensis* tea, with the most used types being black and green tea. The process involves fermenting a symbiotic culture of bacteria and yeast (SCOBY) in a nutritious medium for approximately 7 to 10 days (*1*). During the fermentation process, the SCOBY consumes the sugar in the tea and produces a range of organic acids and enzymes, giving the kombucha its tangy and slightly sweet taste. As the fermentation progresses, a new layer of SCOBY forms on the surface of the liquid and can be used to start a new batch of kombucha (*2,3*). The process leads to the production of organic acids that increase their concentration in the broth, resulting in a decrease in its pH value. This pH drop causes a colour change in the broth, which is attributed to modifications in the phenolic compounds (4). Enzymatic activity on polyphenols is also believed to contribute to this colour change (*5*).

Due to the presence of various strains of bacteria and yeast, the fermentation of kombucha results in the production of acetic acid, ethanol, lactic acid, gluconic acid, and

glucuronic acid, as well as a large amount of phenolic compounds (*5,6*). The microorganisms associated with kombucha production are called chemoheterotrophs, as they derive energy and carbon from the decomposition of organic matter (*7*). Sucrose is one of the primary sources of carbon used in the kombucha fermentation process; however, it is not completely utilised during the process (*8*). Green tea is the most used for kombucha fermentation due to its better stimulant effect than other teas with the same parameters, resulting in a shorter production time (*9*).

The SCOBY is primarily composed of cellulose produced by acetic acid bacteria, which exhibits high water retention capacity, crystallinity, and thermal stability (*10*). Additionally, it possesses characteristics such as biodegradability, purity, and biocompatibility, which opens possibilities for its application in various fields (*11*). Despite being chemically equivalent to plant cellulose, bacterial cellulose (BC) does not contain any by-products such as lignin, pectin, hemicellulose, or other constituents of lignocellulosic materials, making it a chemically pure form of cellulose (*12*). This makes BC a highly versatile material that finds applications in multiple industries, including the food industry, biomedical, pharmaceutical, cosmetic, and even bioengineering (*13*). Furthermore, one major challenge is making BC more appealing to the market since its current production is characterised by low efficiency, long production time, and high cost (*14)*.

As a result, several studies are being carried out with a focus on using industrial waste as an alternative substrate for BC production. These waste materials may contain nutritional sources that can be used as an energy source by bacteria, thus reducing production costs. Furthermore, using industrial waste for BC production can help address waste management issues with less environmental impact compared to conventional raw materials (*15*).

Coffee is one of the most consumed beverages in the world, and its worldwide consumption is expected to reach the equivalent of 10.2 billion kilograms of coffee in 2023 (*16*). Coffee grounds are rich in caffeine, amino acids, phenolic compounds, minerals, and polysaccharides, making them a promising source to produce BC. Furthermore, using coffee grounds residue as an alternative substrate can reduce the volume of waste generated by the coffee industry, contributing to waste management and environmental preservation (*17*).

In this context, this study was carried out to optimise the production of bacterial cellulose through experimental designs. The justification for this work is the importance of optimizing bacterial cellulose production to make it more efficient and sustainable, contributing to the reduction of costs and environmental impacts. In addition, bacterial cellulose is a versatile material with various applications in industry, making optimizing its production a

critical strategy for promoting sustainable development. To achieve this objective, the Plackett-Burman design was used to identify the significant variables for bacterial cellulose growth, followed by the Box-Behnken design to establish the most optimized culture medium.

MATERIALS AND METHODS

Microorganisms and raw materials

The starter culture (SCOBY) was obtained from a local source in the Curitiba region of Paraná, Brazil, and was maintained in a standard medium (70 g/L of sucrose and 3 g/L of green tea) in a bacteriological incubator at 28 ± 2 ºC. PA grade sucrose (Neon, Suzano, Brazil) was used. The green tea and vitamin complex were purchased commercially from a natural products store in Curitiba, Paraná. The coffee grounds residue was kindly obtained from the Research and Development department of Café Iguaçu company in Cornélio Procópio, Paraná, Brazil.

Culture medium selection

For the determination of factors that influence BC production, the Plackett-Burman (*18*) design was used, where seven independent variables were studied at higher (+1) and lower (-1) levels of 8 experiments, including nutrients such as sucrose, green tea, coffee grounds residue, vitamin complex, and kombucha, as well as physical factors such as SCOBY size and incubation *t*. Based on the Plackett-Burman results, the most relevant variables were analysed. Then the Box-Behnken (*19*) design was used to define the most optimized culture medium with three independent variables at levels of -1, 0, and +1, using 15 experiments. The parameter used to determine the best medium for BC growth for both designs was the daily production of BC (g cellulose/day·L), calculated according to the following equation:

$$\text{BC production}=(m_b/t_c \cdot V_c) \qquad /1/$$

where m_b is the dry mass of BC (g), t_c is the cultivation time (days), and V_c is the culture medium volume (L).

Production and treatment of BC

The experiments used autoclaved distilled water to produce 150 mL of culture medium and were incubated in a bacteriological incubator (Vulcan, São Paulo, Brazil) at (28±2) ºC. After determining the optimized culture medium, the membranes were purified with a 0.1 M NaOH solution at 80 ºC for 2 h and washed with distilled water. To determine the production of BC, the purified BC was dried at 50 ºC for 48 h until reaching a constant mass (*20*).

Fermentation kinetics

The kinetics of BC fermentation were evaluated for 7 days using the optimized culture medium formulation, which was determined to be the optimal incubation time for BC production. For the fermentation kinetic evaluation, beakers containing 40 mL of culture medium were placed in a bacteriological incubator (Vulcan, São Paulo, Brazil) at (28±2) ºC, and samples were collected every 24 h in triplicate. BC was collected daily and used to calculate the medium productivity. The fermented medium was then used for pH, total phenolics, total flavonoids, antioxidant activity, reducing sugars, and total protein analysis.

pH and total acidity

The pH analyses were performed for the fermentation kinetics every 24 h, from the initial day until the 7th day of fermentation. A benchtop pH meter (PG1800; Gehaka, São Paulo, Brazil) was used for the measurements. Total acidity was determined through gravimetric titration with previously standardised NaOH 0.1 mol/L, until the endpoint with the phenolphthalein indicator was reached. The concentration of acetic acid was calculated using the following equation:

$$c_{\text{acetic acid}} \cdot V_{\text{acetic acid}} = c_{\text{NaOH}} \cdot V_{\text{NaOH}} \qquad /2/$$

where c is the concentration (mol/L) and V is the volume (L).

Colorimetric analysis

Total phenolic compounds (TPC)

The TPC was determined using the Folin-Ciocalteu colorimetric method (*21*). The absorbance was measured at 765 nm using a spectrophotometer (UV-M51; BEL Engineering, Monza, Italy). Calculations were based on a gallic acid calibration curve (Sigma, São Paulo, SP, Brazil), and TPC concentrations in the samples were expressed in mg of gallic acid equivalent (GAE) per L.

Total flavonoids (TF)

The determination of FT was performed using the aluminium chloride colorimetric method (*22*). The absorbance was measured at 510 nm using a spectrophotometer (UV-M51; BEL Engineering, Monza, Italy). The values were calculated based on a calibration curve obtained with the catechin standard (Sigma-Aldrich, St. Louis, USA), and the results were reported in mg of catechin equivalent (CE) per L.

Antioxidant activity (AA)

The AA was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*23*) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (*24*) methods. The absorbance of DPPH was measured at 517 nm and ABTS at 734 nm using a spectrophotometer (UV-M51; BEL Engineering, Monza, Italy). The values were calculated based on calibration curves obtained with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Acros Organics, Geel, Belgium) and expressed in μM.

Reducing sugars (RS)

RS was determined using the 3,5-dinitrosalicylic acid (DNS) method (*25*). The absorbance was measured at 540 nm using a spectrophotometer (UV-M51; BEL Engineering, Monza, Italy). The calculations were based on a calibration curve obtained with glucose (Neon, Suzano, Brazil) and expressed in g/L.

Total protein (TP)

The determination of total protein was carried out using the Bradford method (*26*). The absorbance was measured at 595 nm using a spectrophotometer (UV-M51; BEL Engineering, Monza, Italy). The calculations were based on a calibration curve obtained with bovine serum albumin (Sigma-Aldrich, St. Louis, USA). Results were expressed in g/L.

Water absorption capacity (WAC)

WAC was determined by immersing the dry membranes in deionised water and storing them until equilibrium was reached (approximately 48 hours) in an incubator (Vulcan, São Paulo, Brazil) at 35 ± 2 °C. Then, the membranes were removed from the water, and excess water was removed from the surface. The mass of the hydrated membranes was measured using an analytical balance (Tecnal, São Paulo, Brazil), and the process was repeated until equilibrium was reached. WAC was calculated according to the following equation (*27*):

$$WAC=(m_h-m_d/m_d)\cdot 100 \quad /3/$$

where m_h is the mass of the hydrated membrane (g), and m_d is the mass of the dried membrane (g).

Crystallinity

The BC samples were analysed using a X-ray diffractometer (XRD-7000; Shimadzu, Kyoto, Japan) at the Multi-User Center for Material Characterisation (CMCM) at the Federal

University of Technology, Paraná, Brazil. The equipment was operated at 30 kV and 30 mA with a scan rate of 2º/min and angles ranging from 5 to 100º (2θ).

Identification of microstructures

The identification of microstructures was performed using Scanning Electron Microscopy (SEM) with a Scanning Electron Microscope (EVO MA 15; Zeiss, Oberkochen, Germany) at the Multi-User Center for Material Characterisation (CMCM) at the Federal University of Technology, Paraná, Brazil. For image preparation, the BC was coated with 90 nm of gold, and the equipment was operated at 20 kV and magnified from 500-5000 times until the membrane reached the maximum heat resistance of the equipment.

Thermal stability

The determination of the thermal stability of the BC was performed by thermogravimetric analysis (TGA) and derivative thermogravimetric analysis (DTG) using the simultaneous TGA/DSC equipment (SDT Q600; TA instruments, New Castle, Delaware, USA). Approximately 4−8 mg of the purified dry bacterial cellulose samples were used in a hermetically sealed aluminium crucible under a heating rate of 10 °C/min up to 600 °C using a nitrogen atmosphere with a flow rate of 50 mL/min.

Statistical analysis

All analyses were performed in triplicate. The data were presented as mean ± standard deviation. Statistical analysis was carried out by analysis of variance (ANOVA), followed by Tukey's test to determine significant differences between samples with a 95 % confidence interval ($p \leq 0.05$), which was represented by superscript letters on the standard deviation values. Statistica 8.0 software (StatSoft, Tulsa, Oklahoma, United States) was used for the Plackett-Burman and Box-Behnken experimental designs and their respective analyses, as well as for all colorimetric, pH and total acidity analyses. OriginPro 2023 software (OriginLab, Northampton, Massachusetts, United States) was applied for the determination of BC crystallinity and thermal stability analyses.

RESULTS AND DISCUSSION

Culture medium selection

For the Plackett-Burman design with eight replicates, each column represents an independent variable, and each row represents an experiment. The levels +1 and -1 represent

the higher and lower levels of the independent variables studied. The results of the Plackett-Burman design on the effects of seven culture variables on the production of BC and dry membrane mass are summarised in Table 1.

Table 1

The Plackett-Burman experimental design was used to define which variables significantly influence BC production. It can be observed that within the studied conditions, the best combination of nutrients and physical parameters was achieved in experiment 8, which showed the highest BC production ((3.21±0.03)/(g/(day·L)) and dry membrane mass ((0.32±0.00) g). From the Pareto chart (Fig. 1), it is possible to identify the variables that significantly affected BC production.

Fig. 1

It was observed that the concentrations of sucrose, kombucha, and SCOBY had a significant positive effect on BC production at a confidence level of $p<0.05$. However, the concentrations of vitamin complex, green tea, coffee grounds residue, and incubation *t* did not show significant effects. These results indicate that these factors may not be substantial for SCOBY production or require further investigation to fully understand their effect on the production process. It is important to note that these results are specific to the current study and may vary depending on the experimental conditions used. Therefore, it is crucial to perform appropriate statistical analyses to evaluate the effect of different factors and optimise the culture medium according to the specific needs of each production process. One possible explanation for the fact that only some factors are significant may be the synergistic effect. The combination of the three relevant factors generates a more significant positive effect than would be observed if each factor were tested individually. This type of synergistic effect is common in complex biological processes.

As evident from the results, the optimal composition of the medium requires higher levels of sucrose, kombucha, and SCOBY. Therefore, only the variables that showed significance according to the Plackett-Burman design were selected for the Box-Behnken experimental design. The Box-Behnken design was carried out with fifteen replicates, where each column represented an independent variable, and each row represented an experiment. The levels +1, 0, and -1 represent the levels of the independent variables under study. The results of the Box-Behnken design on the effects of the three cultivation variables on the production of BC and membrane dry mass are summarised in Table 2.

Table 2

The Box-Behnken design was used to determine the optimal culture medium for bacterial cellulose production. To obtain the best optimisation conditions, the desirability function of the Statistica 8.0 program was used, which allowed for the determination of optimal values for the studied parameters. The individual desirability of each variable was analysed, showing the effect of each variable on the production of BC. The results indicated that the higher levels of sucrose (+1), kombucha (+1), and lower level of SCOBY (-1) were the most significant factors in the optimized culture medium for bacterial cellulose production, resulting in a global desirability of 0.87.

Therefore, based on the ideal levels for BC production, it was possible to obtain the optimized composition of the culture medium, which consists of 110 g/L sucrose, 40 % (*V*/*V*) standard kombucha, 5 % (*m*/V) SCOBY and 3 g/L green tea. These results can be used to optimise the BC production process and improve its efficiency on a large scale.

Evaluation of fermentation kinetics

The kinetic evaluation of the culture medium was used to determine the processes that occurred during kombucha fermentation and bacterial cellulose production. Table 3 summarises the results obtained from TPC, TF, and AA for 7-day kinetics of the optimized culture medium. Table 4 presents the data obtained for concentrations of pH, total acidity, RA, TP, BC production, and mass of dry BC for kinetics of same scope.

Tables 3 and 4

Determination of the TPC, TF, and AA

The fermentation process of kombucha is known to positively influence the concentration of phenolic compounds in green tea, as these compounds are more stable in acidic than in alkaline solutions. Thus, the fermentation process of kombucha can increase the concentration of phenolic compounds in tea (*5*). The findings indicate a significant growth in kombucha production using the optimized culture during fermentation. Specifically, the concentration of phenolic compounds in the fermented tea increased by an impressive 80 % over 7 days.

Chakravorty *et al.* (*6*) demonstrated that black tea fermentation resulted in a 54 % increase in total phenolic compound concentration after 21 days. On the other hand, Özdemir and Çon (*28*) observed an increase of about 20 % in phenolic compounds for green tea kombucha and 10 % for black tea kombucha. Gaggìa *et al.* (*29*) obtained a maximum increase of approximately 35 % on the 7th day of green tea fermentation. It is important to note that

several factors can influence the variation of phenolic compounds in the medium, such as fermentation *t*, origin, and type of tea used, as well as the composition of the starter culture (*30*). However, the optimized culture medium used in the current study showed a superior capacity for phenolic compound production compared to other studies.

At the beginning of fermentation, bacteria and yeasts have greater metabolic activity, which can lead to the degradation of some flavonoids. However, as fermentation progresses, the metabolites produced by bacteria and yeasts begin to influence the activity of enzymes, leading to a higher stability of flavonoids in the medium. In addition, some metabolites produced during fermentation, such as organic acids, can help extract and solubilise flavonoids present in tea (*31*). For these reasons, it is common to observe oscillations in the total flavonoid concentration during kombucha fermentation, with a decrease and subsequent increase.

Although the final concentration of flavonoids was lower compared to other studies, with a total of (51.12±0.10) mg/L, there was a significant increase of 22.5 % at the end of fermentation. In a study carried out by Li *et al.* (*32*), there was a 14 % increase in flavonoid concentration for traditional green tea kombucha, increasing from 318.21 to 362.90 mg/L during fermentation. Jakubczyk *et al.* (*33*) obtained a total concentration of (146.8±3.4) mg/L of flavonoids in 7 days of fermentation. According to Gaggìa *et al.* (*29*), the highest values of flavonoids are observed at seven days of fermentation, where they obtained an 11.6 % increase in green tea medium, with a decrease in longer fermentations.

The AA of kombucha is directly related to the concentration of phenolic compounds and their ability to react with free radicals, and this activity increases over the fermentation *t*. In longer fermentations, a more significant increase in AA can be observed. For instance, in Chakravorty *et al.* study (*6*), the AA of DPPH and ABTS radicals increased by 39.7 % and 38.4 %, respectively, in a 21-day fermentation. However, AA depends on various factors, including substrate type, fermentation *t*, and the microorganisms present. Khosravi *et al.* (*34*) reported a 7 % increase in DPPH radical AA in 15-day fermented black tea kombucha. Chu and Chen (*35*) found a 70 % and 40 % increase, respectively, in DPPH and ABTS radical AA in 7-day fermented black tea kombucha.

The present study evaluated two antioxidant tests, DPPH and ABTS, during seven days of kombucha fermentation. As shown in Table 3, the DPPH AA decreased in the first few days, resulting in a 0.16 % reduction from the first day. However, AA started to increase again

after the 6th day and is expected to continue to rise, indicating that kombucha may be a promising source of antioxidant compounds.

The ABTS AA also decreased in the first few days but increased by 18.85 % at the end of the 7th day. These results suggest that kombucha may have a significant antioxidant effect, especially in longer fermentations. One possible explanation for the initial decrease in AA during kombucha fermentation is that, as mentioned earlier, bacteria and yeast have higher metabolic activity at the beginning of fermentation, which uses phenolic compounds as substrates for their growth. This could lead to the degradation of these compounds and, consequently, a temporary reduction in AA.

However, it is important to note that these initial decreases in AA during fermentation should not be considered negative, as they are a natural and necessary part of the kombucha production process. Moreover, a significant increase in AA can be observed in longer fermentations. AA may also vary depending on the type of test used and other factors, such as the concentration of other bioactive compounds, which can influence the AA of kombucha.

Determination of pH and total acidity

The kinetics of the acidification is a fermentative process that uses a symbiotic culture of bacteria and yeast (SCOBY). During fermentation, the bacteria and yeast present in the SCOBY use the sugar from the tea to produce organic acids, mainly acetic acid and glucuronic acid, which are responsible for acidifying the medium (*6*).

The initial pH of the medium was 2.69 and decreased to 2.38 on the 7th day, indicating that the medium became more acidic during fermentation. There was a sharper decrease in pH between days 2 and 5, and from days 6 to 7, it decreased steadily. According to Jayabalan *et al.* (*36*), after a few days of fermentation, kombucha tends to slow down the decrease in pH due to the buffering effect resulting from the dissociation of carbon dioxide (CO2), which occurs through the reaction between the hydrogen ions (H+) from the organic acids and the bicarbonate ions (HCO3-).

In addition to the acidification of the medium, an increase in total acidity was also observed during fermentation, expressed in grams of acetic acid/100 g. The production of acetic acid is a characteristic of kombucha production due to the presence of acetic acid-producing bacteria in the SCOBY. The total acidity started at 0.40 g/100 mL and reached 1.09 g/100 mL at the end of the 7th day. During longer fermentation periods, there can be a

significant increase in total acidity, indicating a concentration of organic acids. These acids exhibit antimicrobial activity and have an inhibitory effect on acid-intolerant species, such as *Escherichia coli*., *Shigella dysenteriae, Salmonella* Typhi and *Vibrio cholera* (*37*).

Determination of RS

Reducing sugars are carbohydrates with a free aldehyde or ketone group in their structure, which reduce metal ions such as cupric ions (Cu2+) to cuprous ions (Cu+) in alkaline solutions. Glucose and fructose are examples of reducing monosaccharides. In contrast, sucrose, a disaccharide composed of one molecule of glucose and one of fructose, does not have a free aldehyde or ketone group and therefore is considered a non-reducing sugar. However, sucrose can become a reducing sugar under certain conditions, such as enzymatic action or acid hydrolysis, which break down the sucrose molecule into its glucose and fructose components, which are reducing sugars (*38*). This is important to produce kombucha, as the fermentation of this beverage involves breaking down the sucrose in the tea into reducing sugars, which are used by the bacteria and yeast in the SCOBY to produce organic acids and other substances.

Table 4 shows the amount of reducing sugars throughout the fermentation process of kombucha, with an initial concentration of reducing sugars in the medium of 24.77 g/L. The table shows that the breakdown of sucrose into reducing sugars was slower in the first three days and, from the 4th day onwards, occurred more rapidly, reaching 77.05 g/L at the end of the 7th day. This increase in reducing sugars is expected in the production of kombucha, since the beverage is produced by fermenting the sucrose present in the tea.

It is important to note that the final concentration of reducing sugars in the fermentation of kombucha may vary depending on the conditions of the fermentation process, such as the amount of sugar added to the tea and the fermentation *t*. In this study, 40 % (V/V) of previously fermented kombucha was used, which means that the beverage used already contains reducing sugars in its composition, and since sucrose is not completely consumed in the first week of fermentation (*8*), this may explain the relatively high amount of reducing sugars at the end of the fermentation process.

Determination of TP

During the process of kombucha fermentation, there is an improvement in the digestibility of nutrients present in the culture medium. This benefit is attributed to the SCOBY, which is capable of synthesising enzymes such as proteases and lipases that aid in breaking

down proteins and lipids present in the ingredients of the culture medium. As a result, the nutrients are transformed into simpler forms, making them more accessible for microbial growth (*39*).

The kinetics of protein concentration in the culture medium was analysed, and an 18.6 % increase was observed in seven days of fermentation, reaching a final value of 0.70 g/L. This result is in line with the literature, which indicates that the total protein value should be around 1 g/L (*40,41*). It is important to note that the decrease in concentration in the first few days is due to the consumption of nitrogen by microorganisms during the fermentation process (*34*).

Previous studies, such as Kallel *et al.* (*42*), showed that a total protein value of 0.66 g/L was achieved in fermented green tea medium for six days, presenting a growth of 40 %. On the other hand, Sreeramulu, Zhu, and Knol (*31*) found a protein concentration of approximately 0.20 g/L in 14 days of fermentation using a culture of black tea. These differences can be explained by variations in the ingredients of the culture medium, fermentation *t*, and strains of microorganisms used in each study.

Determination of WAC

The BC produced in an optimized green tea medium showed a water absorption capacity of (524.48±9.20) %. This result is consistent with those obtained by other authors when cellulose was produced in a green tea medium, where Vieira *et al.* (*43*) obtained (529±4) %, and Saibuatong and Phisalaphong (*27*) presented a capacity of 490 % for pure BC. However, the composition of the medium is determinant for the variation found between different studies (*43*). Water absorption is an important property of bacterial cellulose, as it directly affects its application in other areas. For example, in biomedical applications, the capacity to retain water is important for maintaining adequate moisture in damaged tissues such as burns or wounds (*44*).

On the other hand, in food applications, water absorption capacity is important to improve the texture and stability of food (*45*). Additionally, water absorption can also affect the mechanical properties and porosity, which can be useful in applications such as filtration membranes and support for enzymatic reactions (*46*). Therefore, understanding and controlling the water absorption capacity of bacterial cellulose is crucial for its application in different fields.

Identification of microstructures

The Fig. 2 show SEM top-view micrographs of the morphology of the BC produced in the optimized medium. The BC showed a smooth and uniform surface without large visible sediments. The absence of visible sediments on the surface of the BC indicates a high degree of purity, which is a desirable characteristic for various applications. Additionally, the smooth and uniform surface of the BC is an important factor for its potential use as a scaffold for tissue engineering, as it can provide a suitable environment for cell adhesion and growth. These results demonstrate the effectiveness of using an optimized medium for BC production and highlight the importance of controlling the production parameters to achieve the desired physical and chemical properties of the material.

Fig. 2

Determination of crystallinity

Fig. 3 presents the X-ray diffractogram of the optimized BC. The peaks observed in the diffractogram correspond to the reflections of the crystalline planes of cellulose, which are indicative of the crystalline structure of the BC. The crystalline structure of BC is composed of long chains organised into fibrils that form crystalline layers intercalated with amorphous regions.

The peaks at 14.48º and 22.74º can be observed for the optimized BC. These peaks were similar to the results presented in the research of Fernandes *et al.* (*47*) for BC produced in green tea with peaks at 14.6° and 22.7°. In addition, the study by Vieira *et al.* (*43*) also showed that the BC membrane in green tea presented two peaks at 15.0° and 22.4°, confirming that these are characteristic peaks of the crystallinity of bacterial cellulose.

The crystallinity index of BC was 79.82 %. This value was higher than that of other studies, where Fernandes *et al.* (*47*) obtained a crystallinity index of 53 % for pure BC. Vieira *et al.* (*43*) achieved a crystallinity index of 60 % for BC produced in green tea medium. These results demonstrate the relevance of producing bacterial cellulose in an optimized medium for obtaining a material with high crystallinity, which can have significant implications for future applications.

Fig. 3

Determination of thermal stability

DTG analysis revealed the existence of three distinct decomposition peaks shown in Fig. 4. The first peak, observed around 49 °C, can be attributed to moisture loss on the membrane surface. The second peak, observed around 188 °C, can be associated with the

decomposition of hemicellulose present in the sample, which is one of the main non-cellulosic components of the bacterial cell wall. The third peak, observed around 336 °C, can be associated with the decomposition of cellulose itself. This third peak is considered an indicative of the thermal stability of bacterial cellulose, as cellulose decomposition occurs at high temperatures and can be affected by different factors such as the presence of impurities or adsorption of other compounds. The literature suggests that the peaks observed between room temperature and 200 °C are attributed to the presence of water and other substances (*48*), while the peak occurring at temperatures above 300 °C is a result of the decomposition of BC membranes (*49*).

Fig. 4

The results of the TGA and DTG analyses present similar behaviours. In the case of TGA, declines in the TGA curve were observed at three distinct temperature points: 152, 267 and 359 °C. These declines represent the decomposition of BC, starting at 152 °C, with a loss of 5.26 % of mass, followed by another decline up to 267 °C, with a loss of 18.77 % of mass, and finally, the third decline up to 359 °C, with a loss of 50.39 % of mass.

According to the analyses performed, it was found that BC presents high thermal stability at elevated temperatures, which may indicate great potential for its application in the packaging industry. Potentially, BC can withstand the temperatures used in packaging sterilisation processes, which range from 135 to 150 °C in a high heat treatment (*50*). However, further studies are needed to evaluate whether the phenolic compounds present in the adsorbed BC would remain stable after such procedures to determine its viability as an active packaging.

CONCLUSIONS

The results of this study demonstrate the successful optimisation of a culture medium for bacterial cellulose production, as well as the characterisation of the resulting BC membrane and the kinetics of its fermentation. During fermentation, the total phenolic and flavonoid contents of the medium increased significantly, and a high antioxidant capacity was observed. These findings suggest that kombucha produced using this optimized formulation could serve as an important source of bioactive compounds for human consumption. Additionally, the BC membrane exhibited a high-water absorption capacity, indicating its potential for use in liquid retention applications. These results have implications in various sectors, including the food and pharmaceutical industries, contributing to the development of healthier and more sustainable products. Therefore, the optimized formulation has excellent potential for the

production of a kombucha-like beverage with a high concentration of bioactive compounds, as well as for achieving a more productive and efficient production of bacterial cellulose. The BC also has potential applications in packaging due to its high thermal stability during sterilisation processes, alongside its high adsorption capacity, making it a promising candidate for active packaging use.

While the study did not yield relevant data showing coffee grounds residue to be a growth enhancer for bacterial cellulose, it is worth noting that they exhibit other interesting properties, such as antioxidant and antimicrobial activity. Therefore, it is important not to overlook their potential. However, further research is required to thoroughly investigate their characteristics and properties and evaluate their potential for bacterial cellulose production.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Nicole Folmann Lima contributed as the main researcher and was responsible for the design of the work, data collection, data analysis and interpretation, performing the analysis, drafting the article, and conducting research. Isabela de Andrade Arruda Fernandes helped with the analysis, research, and critical revision of the manuscript. Charles Windson Isidoro Haminiuk and Giselle Maria Maciel served as advisors and provided oversight for the research, contributed to critical revisions, and gave final approval for the manuscript to be published.

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Table 1. Plackett-Burman design for seven variables and results for BC dry mass and daily production

Experiment	X_1	X_2	X_3	X_4	X_5	X_6	X_7	*m*(BC)/g	BC production/(g/(day·L))
1	6	3	500	70	5	10	10	$(2.30\pm0.22)^{ab}$	$(0.23\pm0.02)^{cd}$
2	6	3	1000	70	2.5	7	20	$(1.94\pm0.00)^{a}$	$(0.28\pm0.00)^{de}$
3	6	5	500	50	5	7	20	$(1.48\pm0.09)^{a}$	$(0.21\pm0.01)^{bc}$
4	6	5	1000	50	2.5	10	10	$(1.00\pm0.02)^{a}$	$(0.10\pm0.00)^{a}$
5	12	3	500	50	2.5	10	20	$(1.82\pm0.45)^{a}$	$(0.18\pm0.04)^{a}$
6	12	3	1000	50	5	7	10	$(0.86\pm0.14)^{a}$	$(0.14\pm0.00)^{ab}$
7	12	5	500	70	2.5	7	10	$(1.33\pm0.63)^{a}$	$(0.20\pm0.07)^{a}$
8	12	5	1000	70	5	10	20	$(3.21\pm0.03)^{a}$	$(0.32\pm0.00)^{e}$

X_1=coffee grounds residue (g/L), X_2=green tea (g/L), X_3=vitamin complex (mg/L), X_4=sucrose (g/L), X_5=SCOBY size (*m*/*V*), X_6=incubation time (day), X_7=kombucha (*V*/*V*)

Table 2. Box-Behnken design for three variables and results for BC dry mass and daily production

Experiment	X_1	X_2	X_3	*m*(BC)/g	BC production/(g/(day·L))
1	70	20	7.5	(2.83±0.11)^{a}	(0.40±0.02)^{a}
2	110	20	7.5	(3.44±0.04)^{ab}	(0.49±0.01)^{ab}
3	70	40	7.5	(3.43±0.12)^{ab}	(0.49±0.02)^{ab}
4	90	40	7.5	(5.34±0.16)^{e}	(0.76±0.02)^{e}
5	70	30	5	(3.53±0.12)^{b}	(0.50±0.02)^{b}
6	110	30	5	(4.71±0.14)^{de}	(0.67±0.02)^{de}
7	70	30	10	(3.18±0.07)^{ab}	(0.45±0.01)^{ab}
8	110	30	10	(4.37±0.14)^{d}	(0.62±0.02)^{d}
9	90	20	5	(4.55±0.08)^{d}	(0.65±0.01)^{d}
10	90	40	5	(6.45±0.30)^{f}	(0.92±0.05)^{f}
11	90	20	10	(4.26±0.25)^{cd}	(0.61±0.04)^{cd}
12	90	40	10	(4.60±0.18)^{d}	(0.66±0.03)^{d}
13	90	30	7.5	(3.71±0.15)^{bc}	(0.53±0.02)^{bc}
14	90	30	7.5	(3.40±0.09)^{ab}	(0.49±0.01)^{ab}
15	90	30	7.5	(3.23±0.18)^{ab}	(0.46±0.03)^{ab}

X_1=sucrose (g/L), X_2=kombucha (*V*/*V*), X_3=SCOBY size (*m*/*V*)

Table 3. Total phenolics (TPC), total flavonoids (TF) and antioxidant activity (AA) of the culture medium during fermentation

Day	*γ*/(mg/L)		AA/μM	
	TPC	TF	DPPH	ABTS
0	(371.75±13.26)^{a}	(41.72±0.69)^{a}	(945.00±6.01)^{a}	(2346.75±0.02)^{a}
1	(387.63±15.91)^{a}	(41.21±0.77)^{a}	(942.75±7.77)^{ac}	(2451.00±0.08)^{a}
2	(416.75±22.10)^{ab}	(40.60±0.81)^{a}	(926.12±6.54)^{abc}	(2460.00±0.00)^{a}
3	(474.25±15.03)^{bc}	(40.42±0.09)^{a}	(923.75±4.59)^{abc}	(2306.25±0.00)^{a}
4	(500.50±0.88)^{c}	(40.25±0.40)^{a}	(919.25±1.06)^{bc}	(2227.25±0.00)^{a}
5	(560.50±22.10)^{d}	(41.15±0.08)^{a}	(913.62±7.60)^{bc}	(2350.75±0.01)^{a}
6	(586.13±12.37)^{d}	(46.92±0.71)^{b}	(936.50±7.77)^{abc}	(2592.50±0.03)^{a}
7	(668.63±5.30)^{e}	(51.12±0.10)^{b}	(943.50±1.76)^{a}	(2789.00±0.01)^{a}

DPPH=2,2-diphenyl-1-picrylhydrazyl, ABTS=2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

Table 4. pH, total acidity, reducing sugars (RS), total proteins (TP), BC dry mass and daily production during fermentation

Day	pH	Total acidity/(g/100 mL)	γ(RS)/(g/L)	γ(TP)/(g/L)	m(BC)/g	BC production/(g/(day·L))
0	(2.69±0.00)^{c}	(0.40±0.00)^{c}	(24.77±0.36)^{a}	(0.59±0.00)^{b}	(0.00±0.00)^{a}	(0.00±0.00)^{b}
1	(2.67±0.01)^{c}	(0.6±0.02)^{a}	(27.43±0.06)^{b}	(0.61±0.00)^{d}	(0.00±0.00) ^{a}	(0.00±0.00)^{b}
2	(2.59±0.01)^{e}	(0.64±0.04)^{a}	(30.07±0.08)^{c}	(0.52±0.00)^{a}	(4.58±0.09)^{b}	(2.29±0.05)^{d}
3	(2.55±0.01)^{d}	(0.69±0.02)^{ab}	(34.97±1.03)^{d}	(0.53±0.00)^{a}	(5.14±0.24)^{bc}	(1.71±0.08)^{c}
4	(2.49±0.01)^{b}	(0.73±0.00)^{ab}	(45.77±0.66)^{e}	(0.53±0.00)^{a}	(5.70±0.48)^{c}	(1.42±0.12)^{a}
5	(2.45±0.00)^{b}	(0.77±0.04)^{b}	(58.94±0.42)^{f}	(0.56±0.00)^{b}	(7.41±0.07)^{e}	(1.48±0.01)^{ac}
6	(2.41±0.01)^{a}	(0.95±0.04)^{d}	(64.40±0.54)^{g}	(0.63±0.00)^{e}	(8.85±0.42)^{d}	(1.48±0.07)^{a}
7	(2.38±0.00)^{a}	(1.09±0.02)^{e}	(76.96±0.44)^{h}	(0.70±0.00)^{f}	(9.83±0.16)^{d}	(1.40±0.02)^{a}

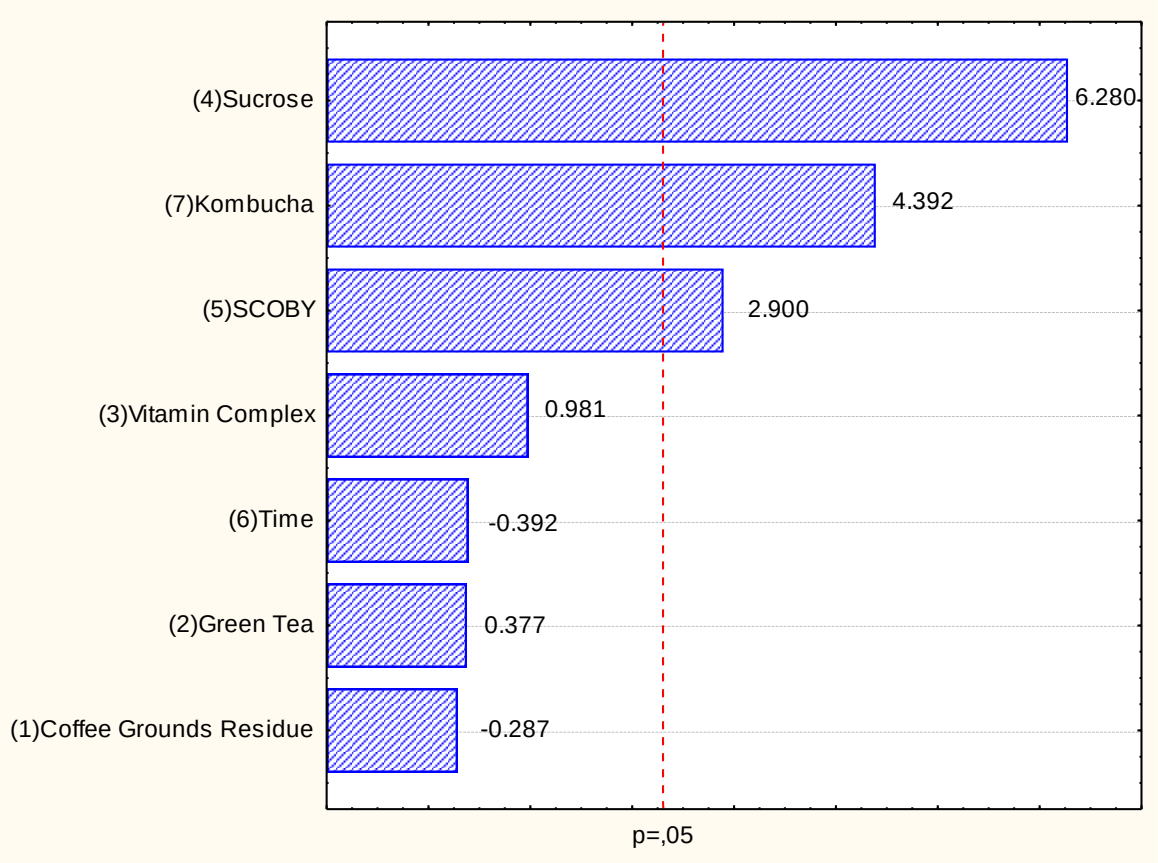

Fig. 1. Pareto chart for the Plackett-Burman design

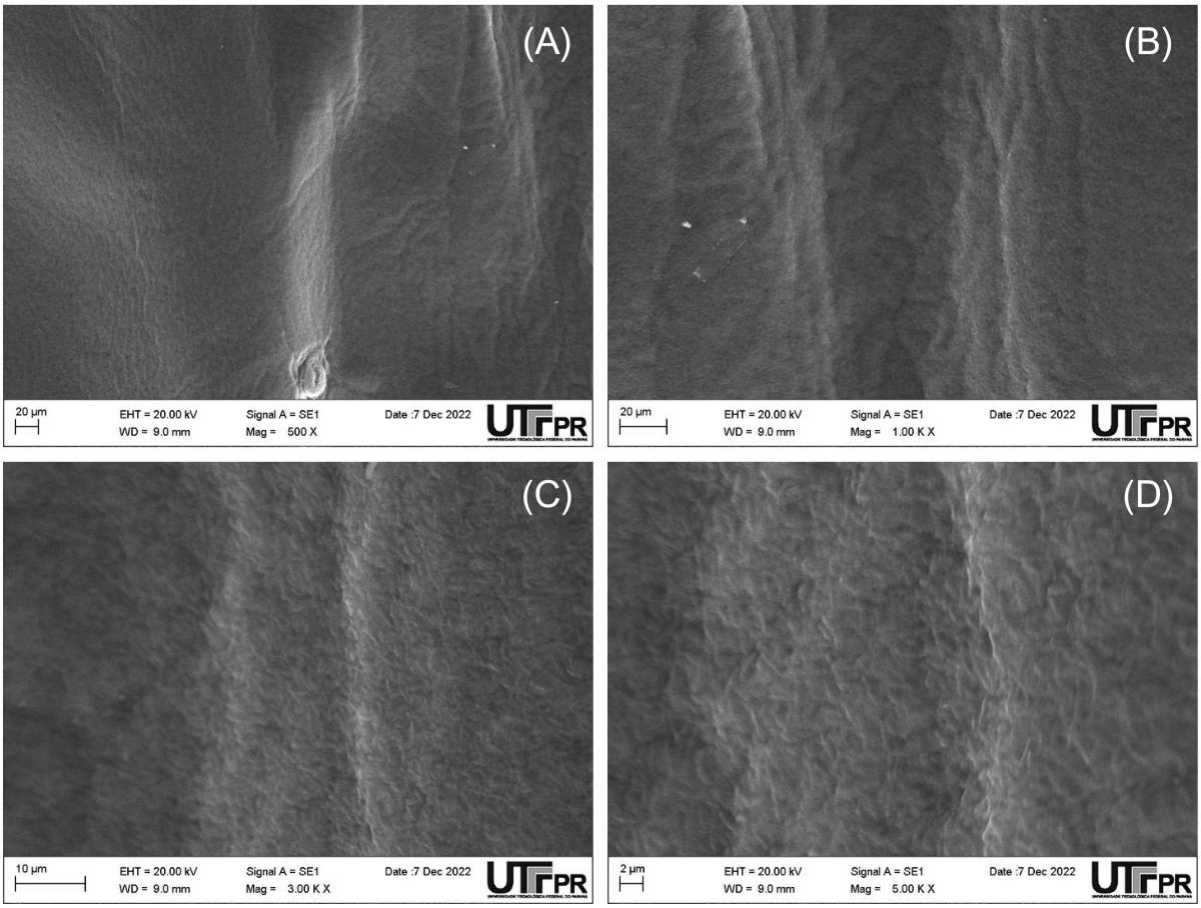

Fig. 2. SEM images of the surface of BC membrane. A) x500 magnification, B) x1000 magnification, C) x3000 magnification, D) x5000 magnification

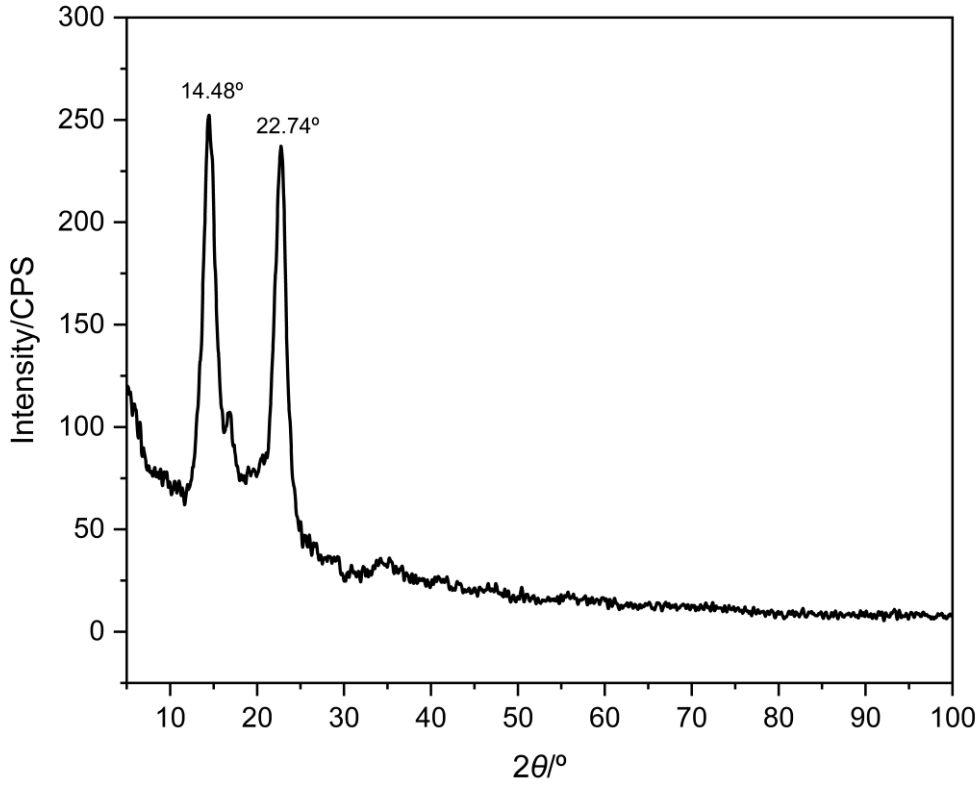

Fig. 3. X-ray diffraction (XRD) diffractogram of purified BC. CPS=counts per second

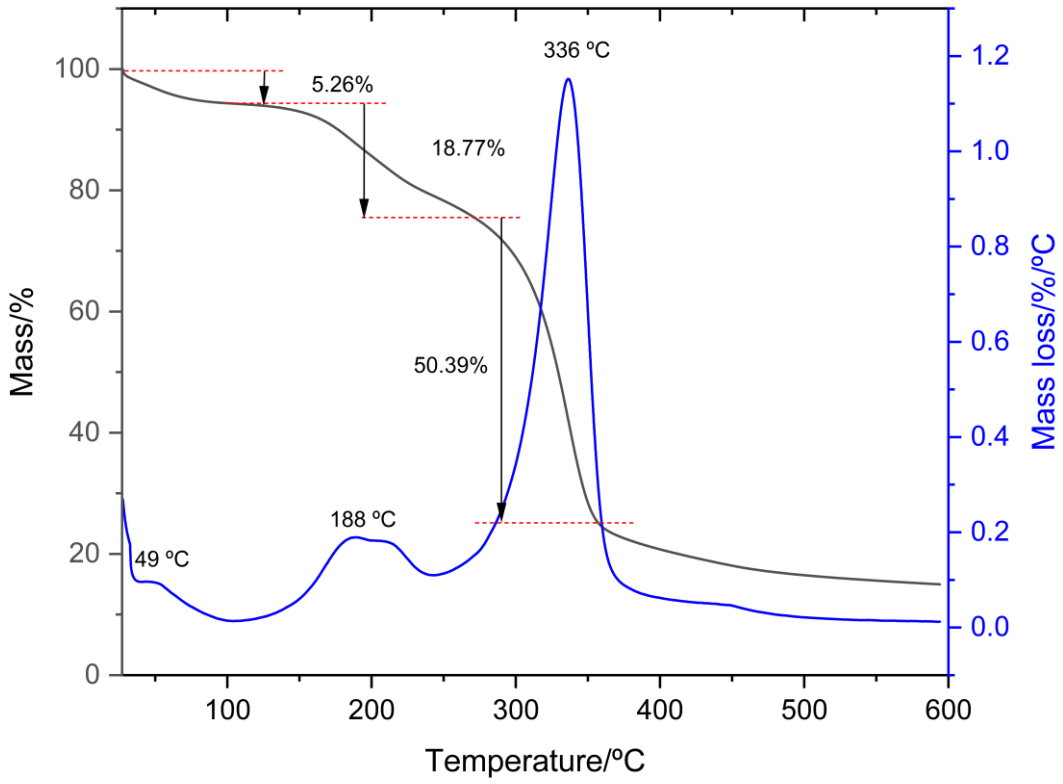

Fig. 4. Thermogravimetric analysis (TGA) and derivative thermogravimetric (DTG) of purified BC