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Bacterial cellulose biosynthesis in the presence of raw moist olive pomace: A green sustainable approach that enhances biopolymer production and properties

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ABSTRACT

In this study, the biosynthesis of bacterial cellulose (BC) by *Komagataeibacter intermedius* strain isolated from Kombucha tea in the presence of raw moist olive pomace (MOP) (concentration up to 40 % in the fermentation media) was studied. The BC membranes were characterized by their antioxidant activity, structural characteristics, crystallinity, thermal stability, and mechanical behavior. Using raw MOP activated the inherent activity of the phenolic compounds, leading to cellular adaptation under unfavorable conditions and increased BC production for all tested MOP concentrations ($p < 0.0001$). This led to a 166.61 % increase for the 20 % MOP group compared to the control (HS medium). For this sample, enhanced antioxidant activity (~40-fold higher than the control) was found, which might be associated with the molecular interactions established between hydroxyls of BC and phenolic compounds. Moreover, an increase of 603.03 % in strain capacity, and a 376.01 % improvement in stress at break compared to the control was observed. The study confirmed that BC can be synthesized using MOP in its natural state, supporting a sustainable circular economy while enhancing the biosynthesis of a value-added product. By reducing synthetic media and utilizing MOP, a greener bioprocess can be achieved, and BC's applicability can be expanded.

1. Introduction

The bioeconomy and sustainable development have driven the biosynthesis of bacterial cellulose (BC) as an innovative and sustainable strategy with promising applications in the biotechnology, biomedicine, food, and agriculture sectors. This encompasses the creation of biopolymers and bio-based nanocomposites, opening new avenues for research and development [1,2]. BC provides undeniable advantages over plant-derived cellulose, including higher crystallinity, greater porosity, and enhanced mechanical and thermal properties. Because it lacks hemicellulose, pectin, lignin, and other biogenic substances, its purity removes the need for extraction processes that involve environmentally harmful chemicals and added costs [3,4].

BC possesses a high-water absorption capacity, which can be attributed to the abundance of hydroxyl groups on its surface. These hydroxyl groups can form hydrogen bonds with water molecules, allowing significant amounts to be absorbed and retained [5]. Although BC and plant cellulose are composed of glucose units in β-1,4-glucan chains, BC fibers have nanometric dimensions, and the incorporation of biomolecules, often *in situ*, can occur during biosynthesis, potentially enhancing biological activity, such as antimicrobial, antioxidant, and anti-inflammatory activity [6]. These features are valuable in the pharmaceutical, cosmetic, and food industries [7]. *In-situ* fermentation of BC with polyphenol-rich by-products from the agro-industry is a technique worth considering for producing naturally polyphenol-fortified materials [8–10].

BC biosynthesis is driven by microorganisms' ability to respond to external stimuli and adapt to harsh environmental conditions. This process enhances their adhesion by forming biofilms and fostering symbiotic relationships within the microbial community through synergistic quorum-sensing interactions [11]. Exposure to stressful stimuli can induce the excretion of polymeric networks as a form of cellular

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protection [12,13]. Consequently, complex nonconventional substrates such as waste or by-products from the agro-industry have been explored as alternative carbon sources to reduce costs and increase production yields, thereby expanding BC's potential applications [14,15].

Given the complexity of nonconventional substrates, physical-chemical pre-treatments are often utilized to obtain carbon-rich substrates, which raises bioprocess costs and may produce environmentally harmful byproducts. Gomes and colleagues [16] assessed dry olive mill residue for BC production with *Gluconacetobacter saccharin*. The process included a step in which the residue underwent aqueous extraction (40 and 100 °C) and acid hydrolysis (H_2SO_4 at 1M) treatments to yield aqueous extracts rich in sugars that can provide energy for the anabolic pathways involved in BC production. In this context, using raw subtracts without any additional treatment is beneficial for process simplicity and economic advantages.

In the olive oil industry, the extensive use of the two-phase continuous extraction centrifugal systems produces significant amounts of moist olive pomace (MOP), a persistent by-product characterized by high moisture content and low viscosity. Typically, MOP is processed into crude pomace oils, with the extracted pomace often burned in boilers to generate energy. This solvent extraction process has detrimental environmental effects. Consequently, finding new applications for MOP will improve circularity in the olive oil sector while addressing environmental concerns. The use of MOP for the biosynthesis of biopolymers, particularly BC, is a novel approach. It provides microbes with nutritional support and induces a biological response due to its high content of phenolic compounds, including oleuropein, hydroxytyrosol, and tyrosol, along with, to a lesser extent, luteolin and apigenin, as reported in previous research [6]. The secretion of microbial exopolysaccharides like BC is linked to the ability to protect cells from external stressors, enhancing their competitiveness and interactions with microbial chemical signals, which can selectively regulate gene expression and trigger the metabolic pathways responsible for exopolysaccharide production.

Bacteria of the genus *Komagataeibacter*, commonly used for BC production, are naturally present in fruit juices and act as fermenting agents in Kombucha tea [17]. *Komagataeibacter intermedius* stands out in the industrial field for its tolerance to acidic conditions and the presence of varying sugar concentrations [18], an essential factor to be considered in new BC synthesis approaches. This facilitates the use of alternative fermentation media, which has potential high revenues, as the substrate accounts for 30 % of the total process cost [19].

This work aims to evaluate the biosynthesis of BC in the presence of different concentrations of MOP by the *K. intermedius* strain CIMO 21LE010 isolated from Kombucha tea and characterize the obtained materials to analyze the impact of its presence and concentration on BC properties. The work is supported by the rationale that introducing raw MOP can induce microbial stress and a consequent cellular response, impacting BC production and properties. Moreover, reducing the use of synthetic medium in favor of MOP can lower process costs. The impact of MOP on BC's final properties can create new opportunities for broadening its range of applications. To the best of the authors' knowledge, this is the first work reporting the use of raw MOP as a fermentation media supplementation to produce BC using *K. intermedius* isolated from Kombucha tea.

2. Materials and methods

2.1. Moist olive pomace samples

The MOP was obtained at the olive oil extraction unit Olimontes (Macedo de Cavaleiros, Portugal), working with a continuous two-phase centrifugation system. The pomace was transported in sterilized plastic containers at +4 °C to the Mountain Research Centre (CIMO), Polytechnic Institute of Bragança, Portugal. The samples were subjected to pasteurization three consecutive times at 70 °C for 30 min (Ecoline 012, Lauda, Königshofen, Germany), then cooled to +4 °C for storage (H8 A1E W, Hotpoint-Ariston, Lisbon, Portugal) until use.

The MOP total phenolic content (TPC), determined using high-performance liquid chromatography (HPLC) with a Diode Array Detector (DAD), amounted to 18315 mg/kg GAE (gallic acid equivalent), in dry-basis, with the phenols primarily classified into three categories: secoiridoids (oleuropein), which constitute approximately 78 % of the TPC, phenolic alcohols (hydroxytyrosol and tyrosol) at around 19 %, and flavonoids (luteolin and apigenin) at approximately 2 %, as previously reported [6].

2.2. Microorganism and inoculum preparation

2.2.1. Isolation and identification of the BC-producing bacterium

The bacterium was isolated from kombucha tea by spreading 0.1 mL onto GYC medium (50 g L^{-1} glucose, 10 g L^{-1} yeast extract, 15 g L^{-1} $CaCO_3$, 9 g L^{-1} bacteriological agar) with 0,1 % de cycloheximide, followed by purification on Hestrin–Schramm (HS) agar medium (20.0 g. L^{-1}glucose, 5.0 g.L^{-1}soya peptone, 5.0 g L^{-1} yeast extract, 2.7 g L^{-1} sodium hydrogen phosphate, 1.15 g L^{-1} citric acid and 8 g L^{-1} agar, pH 5.8) [20]. The colony was evaluated for purity and morphology, and the bacterium for cell morphology and Gram type by classical microbiological methods. For BC production evaluation, a loop full of cells was transferred to HS broth and incubated for 5 days at 28 °C in static condition. The formed pellicle on the air/liquid interface of the culture broth confirmed cellulose production. The isolated culture was preserved in 30 % glycerol at −80 °C in CIMO culture collection (CIMOCC) with the accession number CIMO 21LE010.

The isolate was molecularly identified by sequencing the partial 16S rRNA gene. The genomic DNA was obtained from a 48-h pure culture on HS medium using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research). The 16S rRNA gene was amplified from the gDNA using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR program, using a thermal cycler MyCycler T100 (Bio-rad), was as follows: initial denaturation 94 °C for 2 min; denaturation 95 °C for 10 s, annealing 50 °C for 20 s, extension 72 °C for 1 min (30x); and final extension 72 °C for 5 min. The PCR mixture volume was 50 μL containing 25 μL of DFS-Taq Master Mix 2x, 0.4 μM of each primer, and 2 μL of DNA template. The PCR product was cleaned with the cleanup kit VWR® ExoCleanUp FAST and sequenced by Sanger (ABI 3730xl, Applied Biosystems, STAB VIDA Lda, Caparica, Portugal) in both directions using the same primers.

The resulting sequences were analyzed with the software BioEdit, and a consensus sequence was obtained using the program Sequencher 4.0. The consensus sequence was compared with those from the database GenBank from NCBI (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), using the BLAST (Basic Local Alignment Search Tool) algorithm. The 16S rRNA gene sequences of the type strains of each species in the *Komagataeibacter* genus were retrieved from the bacterial database BacDive (https://bacdive.dsmz.de/) and aligned with the test strain using Mega11 [21]. Genetic relationship analysis was conducted using the same program. Maximum-likelihood phylogenies were constructed using the Tamura–Nei model [22] and 1000 bootstrap replicates.

2.2.2. Inoculum preparation

For the pre-inoculum, Hestrin-Schram (HS) culture broth was used [20]. Bacterial activation was carried out with a 10 % inoculum (1.5×10^8 cells.mL^{-1}) in a static culture for 7 days at 28 °C (SLN 240, POL-EKO, Wodzisław Śląski, Poland).

2.3. Bacterial cellulose biosynthesis

The BC was synthesized using fermentation media with different MOP concentrations (0 % (control), 1 %, 5 %, 10 %, 20 %, 30 %, and 40 % (w/v)). In summary, on a 100 mL basis, the needed amount of MOP

was added to a sterilized flask (250 mL) to achieve the desired concentrations and then brought to a total of 90 mL with HS broth. Finally, the medium was inoculated with 10 mL of the pre-inoculum (10 %). The control comprised 90 mL of HS broth (0 % MOP). These specific concentrations were chosen based on previous studies and the need to explore a range of conditions. The different study groups, tested in triplicate, were incubated under static conditions for 7 days at 28 °C (SLN 240, POL-EKO, Wodzisław Śląski, Poland). As the concentration of MOP increased, the concentration of HS medium was lower in the different study groups.

2.4. BC purification and quantification

The obtained cellulose membranes were purified by washing with 0.5 N NaOH (JMGS, Portugal) under 50 rpm agitation for 1 h at 28 °C (ES-20/80, BioSan, Latvia), followed by washing with sterile distilled water until a neutral pH was obtained. This alkaline treatment aimed to remove bacterial cells and other organic compounds left over from cell metabolism after fermentation. Afterward, the final membranes were dried in an oven at 30 °C (ED115, Binder, Tuttlingen, Germany) for water elimination until a constant weight was achieved to determine, gravimetrically, BC production. Subsequent characterization was performed on the obtained purified and dried BC materials, which, for simplification, will be designated as "BC samples."

2.5. Bacterial cellulose characterization

2.5.1. Antioxidant activity

The antioxidant activity of the BC samples produced using different MOP concentrations was quantified by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test, according to Aylanc and collaborators [23]. Cellulose membranes (10 mg), cut into small pieces (<4.0 mm), were placed in test tubes, mixed with 1 mL of DPPH methanolic solution (6×10^{-5} M), and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a UV–Vis spectrophotometer (V-730 UV–visible, Jasco, Madrid, Spain). All analyses were performed in triplicate, and the results were expressed as a percentage of inhibition according to the Equation.

$$Inhibition\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the DPPH solution, and A_{sample} is the absorbance of the membranes in DPPH solution.

2.5.2. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed on BC samples using an ABB MB 3000 spectrometer (ABB, Zurich, Switzerland) operating in attenuated total reflectance (ATR) mode using a cell equipped with a diamond crystal. The spectra were obtained between 4000 and 500 cm^{-1} by averaging 32 scans at a resolution of 4 cm^{-1}. The data were acquired and treated using the Horizon MB v.3.4 software. To quantify the crystallinity of the synthesized BCs, the absorbance ratio A1375/A2900, known as Nelson's crystallinity index [24], was determined.

2.5.3. Thermogravimetric analysis (TGA)

The thermal stability of BC samples was analyzed using a NETZSCH equipment (TG 209 F3 Tarsus, Selb, Germany). For each BC sample, 7 mg were weighed into alumina crucibles and heated from 25 °C to 800 °C at 10 °C/min under an inert nitrogen atmosphere with a 50 mL/min flow rate. Thermograms were treated with Netzsch Proteus thermal analysis software, version 5.2.1.

2.5.4. Mechanical properties

The mechanical properties BC samples were analyzed using a tensile tester (Autograph AGS-X Series, Shimadzu, Kyoto, Japan) equipped with a 10 kN load cell and mechanical clamps for fixing the samples. For each sample, 5 replicates were tested with dimensions of 10 mm × 30 mm and a test speed of 5 $mm \cdot min^{-1}$. The stress and strain at break were determined from stress-strain curves.

2.6. Statistical analysis

The results were analyzed using the ANOVA statistical test with Tukey's multiple comparison post-test using the GraphPad Prism® 8.0 software (San Diego-CA, USA).

3. Results and discussion

3.1. Isolation and identification of the BC-producing bacterium

Colony morphology was circular, light beige, with a translucid halo in GYC, denoting acid production. The cells were single Gram-negative rods, typical of acetic acid bacteria. In HS broth, the bacterium produced a visible pellicle at the interface liquid-air. Based on the partial 16S rRNA gene sequence, the bacterial strain CIMO 21LE010 was identified as *Komagataeibacter intermedius*. Fig. 1 shows that the strain clustered with other 'cellulose-producing' species and showed 100 % identity with *K. intermedius* DSM11804^T. The phylogenetic relation among all type strains of *Komagataeibacter* species, based on the partial 16S rRNA gene is shown in Fig. 1.

Komagataeibacter intermedius is often compared to *K. xylinus*, a species highly studied for its substantial BC production under varied conditions. Lin and co-workers [26] reported that *K. intermedius* and *K. xylinus* exhibited similar BC production capability at pH 4 and 5 in the short-term cultivation (4 days). However, *K. intermedius* produced more BC than *K. xylinus* in the pH 6–8 range. Reports have shown that *K. intermedius* can produce higher amounts of BC than *K. xylinus* in multiple substrates, free from impurities, with a high crystallinity index and similar mechanical properties to the one produced by *K. xylinus* [27–29].

3.2. BC biosynthesis quantification

Using fermentation media supplemented with MOP for BC biosynthesis led to promising results, as shown in Fig. 2, where the effect of MOP concentration in BC production can be perceived. MOP, a residual by-product of the olive oil industry in its raw state, demonstrated the ability to enable BC production in all the study groups, with a progressive increase in biosynthesis up to 20 % MOP. The highest achieved BC production was 6.140 g/L, with a significant increment of 166.61 % relative to control (2.303 g/L). The groups 30 % and 40 % MOP gave rise to an average BC production of 5.541 and 3.936 g/L, respectively, with a significant increase ($p < 0.0001$) compared to the control. In conclusion, the 20 % MOP sample is the most favorable option regarding BC production yield. Although higher MOP concentrations resulted in a lower BC production increase, they may still be beneficial due to decreased reliance on synthetic media with consequent cost minimization. For instance, the 40 % MOP sample achieved a 70.908 % BC increase compared to the control while reducing the use of HS medium by 50 %. The decrease in BC production when 30 and 40 % MOP was used can be due to the increased concentration of phenolic compounds in the fermentation medium and associated antibacterial activity inhibiting the anabolic enzymatic action. Among them, flavonoids inhibit nucleic acid synthesis, plasma membrane functions, and energy metabolism [30,31].

According to Distler and co-workers [17], reusing agro-industrial residues as microbial nutritional supplements can decrease costs and make BC biosynthesis viable. They assessed BC biosynthesis using pre-treated sulfite from the paper and pulp industry as a sustainable carbon source for *K. intermedius*. Under static culture conditions and incubation at 30 °C for 7 days, a production of 5.68 g L^{-1} was achieved,

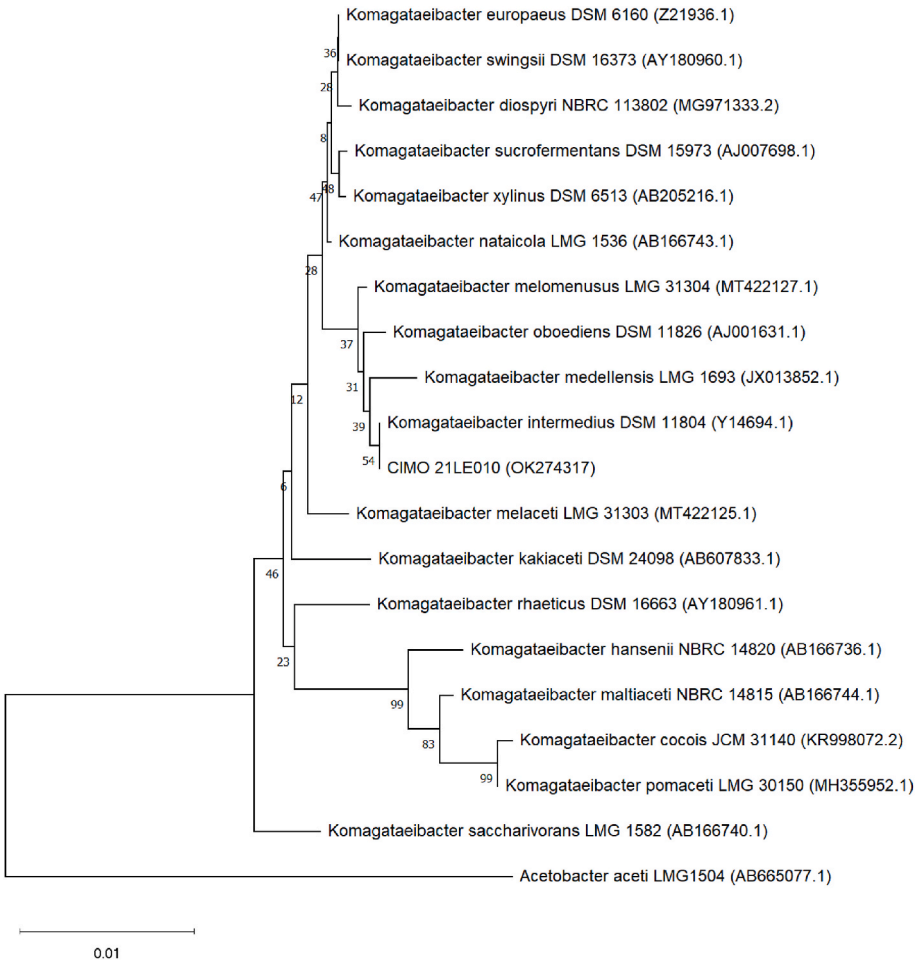


Fig. 1. The genetic relationships between the test strain and the type strains of the *Komagataeibacter* genus were inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985). The branch lengths are shown in the same units as those of the genetic distances used to infer the phylogenetic tree. The genetic distances were computed using the Maximum Composite Likelihood method [25] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 20 nucleotide sequences. *Acetobacter aceti* was used as an outgroup to root the tree. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1241 positions in the final dataset. The analyses were conducted in MEGA11 [21].

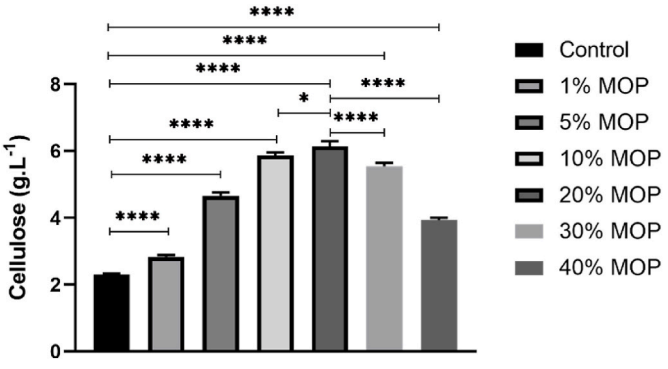


Fig. 2. Amount of BC produced with different MOP concentrations versus the control (BC sample produced without MOP). *p < 0.05; ****p < 0.0001.

corresponding to a BC biosynthesis increment of 13 %. In a study conducted by Gomes and co-workers [32], the possibility of using pre-treated MOP as a carbon source in BC biosynthesis was tested. Following drying, water extraction at 40 °C, and subsequent acid hydrolysis (1M H_2SO_4), MOP was incorporated into the HS medium to replace glucose. After 96 h of incubation of *Gluconacetobacter sacchari* under static culture conditions at 30 °C, a production of 1.28 g L^{-1} was achieved, not reaching the output obtained in the HS control (2.5 g L^{-1}). Both studies reported BC synthesized with supplementation of pre-treated wastes, generating residual byproducts and supplemental bioprocess costs. Moreover, the obtained production levels were lower than those achieved in the present study, highlighting the potential of MOP without any pre-treatment to supplement the fermentation medium.

3.3. BC antioxidant activity

The antioxidant activity of the synthesized BC samples was evaluated. The results are plotted in Fig. 3. In the control group (BC samples produced without MOP), a low DPPH radical scavenging activity was observed, with an average value of 1.09 %, confirming a reduced intrinsic activity of cellulose, an anticipated result based on its molecular composition [33]. The addition of MOP to the substrate showed a significant increase ($p < 0.0001$) in the BC samples' antioxidant activity, with an increasing percentage of inhibition up to 39.13 % for the 20 % MOP sample, which corresponds to a 38-fold increase compared to the control, an effect attributed to the phenolic compounds present in MOP. BC offers significant potential for chemical and/or physical modifications due to the free hydroxyl groups on its surface, facilitating interactions with bioactive compounds that will enhance the biopolymer's

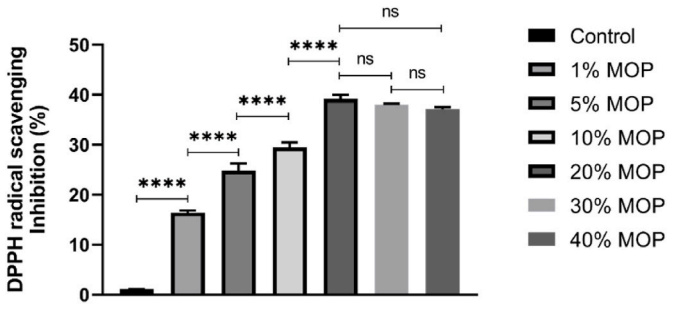


Fig. 3. Antioxidant activity of synthesized samples with different concentrations of MOP and the control (BC sample produced without MOP). ****p < 0.0001; ns = non-significant.

properties. Furthermore, the nanofiber system with crystalline regions makes up a nanoporous network capable of retaining active compounds and forming electrostatic bonds until saturation [34]. This saturation may have led to a stabilized antioxidant activity, as verified by the non-significant observed differences for the 30 and 40 % MOP samples (38.03 % and 37.12 %, respectively). Since a slight decrease trend is observed, it can be hypothesized that the greater availability of phenolic compounds in the fermentation substrate can influence the enzymatic anabolic processes, reducing the incorporation of *in situ* phenolic compounds into the synthesized nanofibers. In fact, the evolution of the antioxidant activity from 20 % onwards can depend on the two previously described phenomena. This verification was not conducted due to the accentuated decreased BC production after 20 % MOP sample. The results obtained when using MOP must be emphasized due to the added value of the formed materials with antioxidant activity.

In a study by Li and collaborators, the efficiency of *in situ* synthesis of biologically active BC was demonstrated through the use of wine pomace and its hydrolysate. They found that BC synthesized from wine pomace hydrolysate exhibits a slower release rate of phenolic compounds, achieving a higher antioxidant activity (~40 % DPPH radical scavenging activity) [35]. It is essential to highlight the achievement of an antioxidant activity value similar to this study, which can be supported by the fact that MOP is an aqueous-rich substrate (73 % moisture content) [36], providing a favorable environment for a slow release of the phenolic compounds up to a concentration of 20 % MOP.

3.4. BC structural characteristics and crystallinity index

Cellulose type I is the most abundant form in nature and is predominantly produced by bacteria [37]. The FTIR spectra of the obtained BC samples are shown in Fig. 4. All study groups presented the characteristic cellulose bands. These comprise a broad band between 3000 and 3700 cm^{-1}, attributed to the hydroxyls (OH) stretching, a band centered at ~2900 cm^{-1} assigned to the aliphatic C-H stretching typical of type-I cellulose [38], and a band ~1640 cm^{-1} attributed to the absorbed water H-O-H stretching absorbed by cellulose. (van Zyl and Coburn, 2019). The absorption bands identified at wavenumbers ~1375, 1170, and 1060 cm^{-1} are attributed to scissoring vibrations of C-H, oscillating vibrations of OH, and skeletal vibrations of C-O-C in the pyranose ring, respectively [39].

Among the registered bands, the absorption around 1450 cm^{-1} is attributed to the symmetric bending vibration of CH_2, known as the 'crystallinity band' [35], which is less evident in BC produced with 20 % MOP. To quantify the synthesized BC's crystallinity degree, the absorbance ratio A_{1375}/A_{2900}, known as Nelson's crystallinity index [24], was determined, and the results, expressed in percentage, are given in Table 1. For all samples, the crystallinity index was greater than 85 %, which agrees with the literature values for BC [40]. Bacterial cellulose has been reported to have a crystallinity greater than 80 %, while vegetable cellulose contains more amorphous regions and exhibits

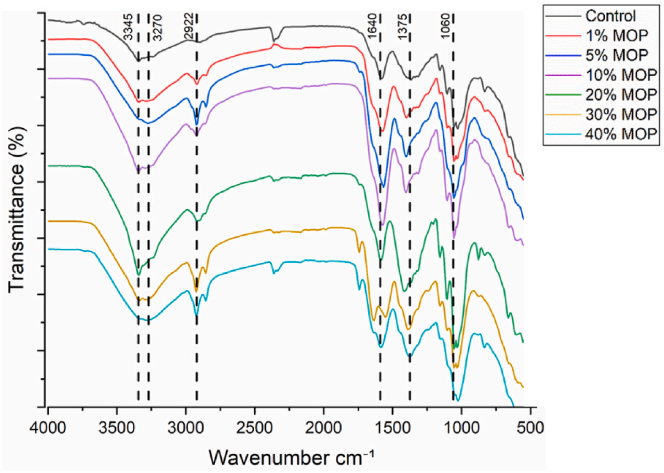


Fig. 4. FTIR spectra of BC samples synthesized with different concentrations of MOP and the control (BC sample produced without MOP). The vertical black dotted lines represent the characteristic peaks of BC.

crystallinity values ranging from 40 to 85 % [41]. The lowest ratios were obtained for the 10 and 20 % MOP groups, 86.05 % and 85.33 %, respectively. The greatest crystallinity was achieved for the control group, BC samples produced without MOP (91.89 %). The results show an inverse relationship between the BC degree of crystallinity and the synthesized amount, i.e., samples with higher BC production exhibited a lower crystallinity index.

3.5. BC thermal stability

The results obtained with the thermogravimetric analysis (TGA) and respective derivative thermogravimetry curves (DTG) of BC samples are represented in Fig. 5. All samples exhibited a thermal degradation profile with multiple stages, highlighting the three mass loss events typical of BC [42,43]. The first thermal event (30–150 °C) was attributed to water evaporation or BC dehydration [44]. The decomposition of small-size proteins and/or phenolic compounds associated with cellulose through hydrogen or electrostatic bonds may also occur at temperatures around 150 °C [45]. In the 10 % MOP sample, thermal degradation is visible at 162 °C with a mass loss of 5.09 %, attributed to the decomposition of the incorporated phenolic compounds.

The second event is marked by a significant mass loss attributed to cellulose degradation, including depolymerization and glucose unit decomposition. The extrapolated onset degradation temperature (t_{onset}) and the maximum degradation rate temperature (t_{max}) were analyzed to evaluate the BC samples' thermal stability. The membranes synthesized with 30 and 40 % MOP supplementation exhibited the lowest t_{onset}, 239.1 and 239.4 °C, respectively, followed by the control with 247.1 °C. The highest t_{onset} was found for the 20 % MOP sample, 259.8 °C, followed by the 10 %, 5 %, and 1 % MOP samples with 257.7, 257.5, and 253.3 °C values, respectively. The obtained values demonstrate that the stability of the BC samples synthesized with MOP is consistent with the values reported in the literature [28,46]. Regarding t_{max}, BC synthesized with 20 % MOP presents the highest value, 273.2 °C, with a mass loss of 30.07 %, while for the control, 268.7 °C, a mass loss of 39.21 % was determined. This difference can be attributed to the higher crystallinity index of the control sample. According to Dima and co-workers [47], crystallized cellulose chains are efficient pathways for heat transfer, producing better thermal conductivity and thus decreased stability. However, other structural parameters, such as molecular weight, and fiber orientation, resulting in molecular compaction or disorganization and consequent activation energy spent, can also influence thermal stability [44,48]. Therefore, from 20 % MOP onwards, a progressive decrease in t_{max} was observed (samples with 30 and 40 % MOP), namely

Table 1
Nelson crystallinity index (%) for the synthesized BC samples using different concentrations of MOP (0–40 %).

Study groups	Control	1 % MOP	5 % MOP	10 % MOP	20 % MOP	30 % MOP	40 % MOP
Crystallinity index (%)	91.89	89.05	87.82	86.05	85.93	87.21	88.76

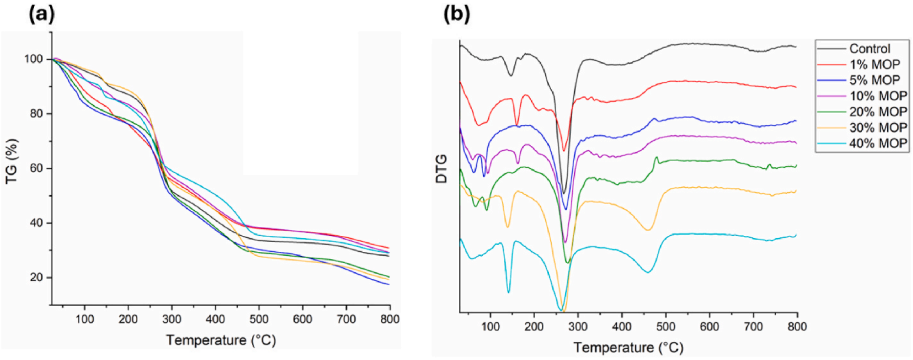


Fig. 5. (a) TGA and, (b) DTG curves of bacterial cellulose membranes produced by *K. intermedius* in different concentrations of MOP and standard HS (control) media.

268.7 °C with a mass loss of 38.9 % and 260.8 °C with a mass loss of 25.02 %, respectively. The results indicate an increase in BC density up to 20 % MOP, probably due to electrostatic forces between the phenolic compounds and a consequent reduction in the membrane porosity. This thermal behavior was not observed in samples with 30 and 40 % MOP, possibly due to the high bacterial stress caused by phenolic compounds, which influences the metabolic pathway of biosynthesis and, consequently, BC production.

The third and final event is compatible with the degradation of carbonaceous residues, with the occurrence temperatures shifting to higher values as the MOP content in the medium increases. The observed residual mass for the control sample was lower (17.96 %) than that of the MOP groups, with contents varying from 18 to 30 %, indicating a greater BC purity for the sample synthesized in the HS medium without MOP addition [48].

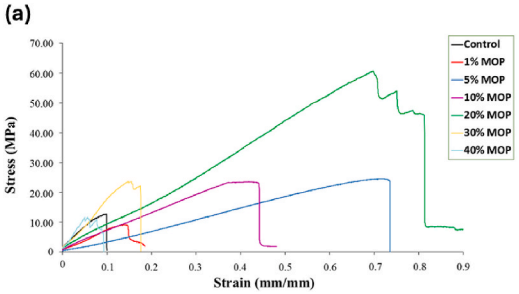
Among all the analyzed samples, the membranes synthesized with 20 % MOP were the ones presenting the higher thermal stability.

3.6. BC mechanical properties

The mechanical behavior of BC samples was investigated to observe the effect of MOP content. The results of the stress-strain curves are presented in Fig. 6a with the determined strength and strain at break shown in Fig. 6b. The results showed variability, which can be associated with the nature of BC, a product of biological synthesis (biomaterial). The membranes produced with MOP showed increased strain at break, except for the 40 % group, which exhibited 65 % lower strain values than the control. The strain at break highlighted the 20 and 5 % MOP groups as the ones holding the better performance, with an increase of 603.03 and 629.29 %, respectively, against the control. The lowest values were observed for the control, and 40 % MOP samples, justified by the higher crystallinity indices. In a study by Chaussé and coworkers [49], from BC membranes synthesized by a new strain isolated from a symbiotic kombucha culture, it was proven that differences in crystallinity inversely affect membrane deformation.

The 20 % MOP samples demonstrated the highest stress at break (60.73 ± 20.29), 376.01 % higher than the control. This result is satisfactory, as a study carried out by George et al. (2005) [50], without the use of residual supplements in the biosynthesis of BC, reported stress at break of 43.68 MPa for membranes obtained from *Acetobacter xylinum* in a medium rich in sucrose and yeast extract, followed by washing with sodium hydroxide sodium. The lowest value was observed for the 40 % MOP sample, which might be associated with the excessive concentration of MOP (phenolic compounds), inducing bacterial stress interfering with the enzymatic anabolic process. Another explanation can be the weak interactions between nanofiber network structures [51].

Overall, the BC's structural properties directly affect the membranes' mechanical properties, depending on the bonding forces established between the molecular chains and the developed hydrogen bonds between the hydroxyl groups [52]. In turn, the micro- and macrostructure of BC are directly influenced by the used biosynthesis parameters, microorganism genus and species, purification method after fermentation, including the used reagent concentration, and the applied drying process [53,54]. It can thus be inferred that the fermentation medium with MOP had a direct action on the mechanical properties of the membranes, giving rise to a satisfactory relationship between



(b)

BC membranes	Stress at break (MPa)	Strain at break (mm/mm)
Control	12.76 ± 40.45	0.10 ± 28.61
1% MOP	9.09 ± 44.42	0.14 ± 22.34
5% MOP	24.56 ± 29.54	0.72 ± 39.33
10% MOP	23.48 ± 40.34	0.43 ± 42.30
20% MOP	60.73 ± 20.29	0.70 ± 36.18
30% MOP	23.67 ± 42.28	0.15 ± 33.34
40% MOP	10.74 ± 52.36	0.06 ± 43.49

Fig. 6. Mechanical behavior of BC membranes synthesized with different concentrations of MOP. (a) Stress-strain curves for one of the 5 replications of BC, (b) average of the BC mechanical parameters.

stress/strain in samples produced with 5, 10, 20 and 30 % MOP, with relevance for the characteristics of 20 % MOP membranes. However, depending on the target application, BC may be biosynthesized with the MOP concentration best suiting the intended purpose.

4. Conclusions

The study demonstrated the possibility of introducing MOP in its raw form in BC biosynthesis, enabling the action of natural bioactive compounds and subsequent metabolic response. This increased BC production was significant for all studied groups ($p < 0.0001$), resulting also in materials with improved properties. It was found that BC synthesized *in situ* with MOP acquire antioxidant activity, with the sample produced with 20 % MOP showing higher productivity (166.61 % relative to the control), superior thermal stability, and greater stress and strain at break. Moreover, a crystallinity index decrease was observed for the membranes synthesized with MOP, even though greater than 85 %.

Reducing the use of synthetic media in favor of MOP has not only improved bioprocess efficiency but also emerged as a strategy to produce BC with enhanced properties, a key factor for strengthening the viability of the BC production chain. Integrating MOP into the circular economy fosters a green and sustainable solution, producing a value-added product with wide-ranging applications in the food, health, and agricultural sectors, mainly targeting biopolymer use and bio-based nanocomposites.

CRediT authorship contribution statement

Pedro J.L. Crugeira: Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization, PEDRO JORGE LOURO CRUGEIRA, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Halima Khelifa:** Resources, Methodology, Investigation. **Luísa M.da S. Barreira:** Methodology, Formal analysis. **Noureddine Halla:** Methodology, Investigation, Formal analysis, Data curation. **António M. Peres:** Investigation, Formal analysis, Data curation, Conceptualization. **Tatiana B. Schreiner:** Resources, Methodology, Data curation. **Maria Filomena F. Barreiro:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Paula Rodrigues:** Writing – review & editing, Supervision, Investigation, Data curation, Conceptualization.

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Data availability

No data was used for the research described in the article.

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