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Alternative production of bacterial cellulose by *Komagataeibacter hansenii* and microbial consortium

Izabel Cristina Nóvak[1], Bruna Segat[2], Michele Cristina Formolo Garcia[2,3], Ana Paula Testa Pezzin[1] and Andréa Lima dos Santos Schneider[2,3]*

[1]Laboratório de Materiais, Programa de Pós-graduação em Engenharia de Processos, Universidade da Região de Joinville – Univille, Joinville, SC, Brasil

[2]Laboratório de Biotecnologia I, Departamento de Engenharia Química, Universidade da Região de Joinville – Univille, Joinville, SC, Brasil

[3]Laboratório de Biotecnologia II, Programa de Pós-graduação em Saúde e Meio Ambiente, Universidade da Região de Joinville – Univille, Joinville, SC, Brasil

**andrea.lima@univille.br*

Abstract

Bacterial cellulose (BC) is a biopolymer produced by several microorganisms and has attracted attention due to its unique characteristics, replacing cellulose extracted from nature. This work aimed to compare different BC production methods and the possible interference of these methods on the characteristics of the BC produced, seeking low-cost and large-scale production. BC membranes were produced by *K. hansenii* and a microbial consortium using different culture media. Rehydration percentage, water-holding capacity, TGA, and FTIR characterized the membranes. The production from the microbial consortium was highlighted for having a higher dry mass yield (0.289 ± 0.199 g), more than triple the amount produced by pure strain. Both samples showed similar chemical structures, as pointed out by FTIR. However, the BC produced by the microbial consortium showed superior thermal stability (357 °C). Moreover, using the microbial consortium, it was possible to obtain BC with a reduction in production cost of 92%.

Keywords: *bacterial cellulose, kombucha microbial consortium, Komagataeibacter hansenii.*

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1. Introduction

Cellulose is one of the most abundant biopolymers on the planet, having plants and wood as its primary sources. Cellulose is present in 33% of all vegetables, reaching 40-50% in wood and up to 90% in cotton[1]. This natural polymer has great economic importance and great technological interest, being considered one of the largest productions in the world, with approximately 19.7 million tons manufactured in Brazil in 2019, according to the 2020 Annual Report of the IBÁ (Brazilian Tree Industry)[2].

However, the global demand for vegetable cellulose (VC) can cause several problems, including deforestation. According to Bologna and Aquino[3], trees store carbon, regulate the water cycle, produce oxygen, contribute to soil conservation, and provide a natural habitat for several species, keeping the atmosphere clean. Another commonly encountered problem is the additional cost generated to remove components such as lignin, hemicelluloses, pectin and other polymers present from the part of interest to the industry. Seeking more sustainable alternatives appears to the production of cellulose through bacteria.

According to Picheth et al.[4], bacterial cellulose (BC) is a biopolymer formed by cellulose microfibrils, intertwined and variable length, forming a translucent and gelatinous membrane, produced extracellularly by genera such as *Gluconobacter*, *Acetobacter*, *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Sarcina*, *Azobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella* and *Alcaligenes*. Among the BC-producing bacteria, the genus *Komagataeibacter* is commonly used for its high yield[5]. In addition, it can convert glucose, glycerol, sugar, or any other organic substances into pure cellulose[6].

Recent studies have sought to understand the biomolecular mechanism of BC production. In this context, Manan et al.[7], funded the involvement of specific operons (bcsABCD), which code for the cellulose synthase (CS) complex. They describe that this operon regulates intracellular biosynthesis, extracellular transport across the cellular membranes, and in vitro assembly of cellulose fibrils into highly ordered structures. Other studies are also moving towards cell-free cellulose production, synthesized in vitro, where you can conduct enzymatic reactions involved in the natural biochemical pathway of cellulose production by microbial cells[8].

The unique properties of BC qualify it for applications in the most diverse areas, such as different food

packaging[9], paper[10], textiles[11] and bioconcrete[12], as well as bioremediation[13], cosmetics[14], electronics[15] and sensors[16] applications. According to Barud et al.[17], BC has excellent potential as a biopolymer, especially in biomedical applications. It will not interfere with the patient's body in the medium and long term. BC's known characteristics are biocompatibility, mechanical resilience, ease of incorporating drugs or nanoparticles, and moistening the injured area while absorbing exudates[18].

BC is chemically equivalent to VC, but it has a high degree of crystallinity and high purity (free of lignin, hemicellulose, pectin and other biogenic components) as well as a unique structure of cellulose nanofiber-weaved three-dimensional (3D)[19]. However, the high cost of the culture medium and low yield are the main challenges in producing BC for industrial-scale applications[20]. Rejected agricultural and industrial waste can be used to synthesize BC through its fermentation. When low-quality fruits are not shipped, many are discarded, and they are rich in glucose and fructose. In this case, they can be used as a carbon source for making useful products, such as BC[21]. Low-cost alternative nutrients are already being researched, such as grape pomace[22], corn steep liquor[23], coconut juice[24] and pineapple[25] from agribusiness, yeast residues from the brewing industry[26], lychee extract[27], among others.

In this context, we also find an alternative fermentation process using a medium composed of an infusion of black or green tea leaves that is fermented with a symbiotic association of bacteria and yeast, which are capable of forming a cellulosic film on the surface of the liquid sweetened product called kombucha[28].

Therefore, in this work, BC production was evaluated using alternative media to analyze the best cultivation condition for membrane formation on a larger scale, using the isolated strain of *Komagataeibacter hansenii* ATCC 23769 and the symbiotic association that gives rise to kombucha.

2. Materials and Methods

2.1 Membrane production

2.1.1 Membrane from *Komagataeibacter hansenii*

The microorganism used was the bacterium *Komagataeibacter hansenii* ATCC 23769, preserved in a liquid medium in a refrigerator. The culture medium used was: Carbon source (20g/L), peptone (5g/L), yeast extract (5 g/L), bibasic sodium phosphate (2.7 g/L) and citric acid (1.15 g/L). Bacterial activation was carried out in Erlenmeyer flasks containing culture medium (pH 7.0) and incubated at 30 °C under static conditions for two days. After this period, the inoculum was transferred to other flasks with culture medium (with the same concentrations already mentioned) at a rate of 20% with initial optical density (O.D.) varying between 0.15 and 0.19. The carbon sources used were mannitol and glucose. Membranes produced by *K. hansenii* using mannitol as a carbon source was named KHMN, while those produced with glucose were named KHGL.

The cultivation of *K. hansenii* was also tested in a culture medium containing black tea (6 g/L) and commercial sugar (50 g/L), boiled for 15 min, filtered and autoclaved before inoculation. The experiments were carried out in different flasks previously sterilized with alcohol, boiling water and exposed to ultraviolet light. Then, the culture media were incubated in an oven at 30 °C and static condition for 12 days to form hydrated BC blankets at the liquid/air interface. These membranes were called KHCP (Figure S1, Supplementary Material).

2.1.2 Obtaining the membrane from the microbial consortium

The prepared cultivation media contained 6 g/L of black tea as nitrogen and 50 g/L of commercial sugar as a carbon source. The inoculum was produced with previously formed and stored membranes, known as SCOBY, for seven days. Together with 100 mL of the remaining broth containing the active symbiotic association, the inoculum membrane was used, inserting them in a new black tea culture medium, with the same composition mentioned, and remaining for 12 days in the flasks for the synthesis of BC. The cultivation was conducted at room temperature and static conditions. These membranes formed at the liquid/air interface were called CMCP (Figure S2, Supplementary Material).

2.2 Purification of membranes

The purification of the membranes was carried out as described by Silveira et al.[29]. Afterward, the membranes were washed with distilled water until reaching pH 7.0, stored in distilled water, autoclaved to avoid contamination, and dried in an oven at 30 °C.

2.3 Membrane characterization

2.3.1 Water retention capacity (WRC)

A never-dried membrane was used, immersed in deionized water. The sample was removed and dried on absorbent paper to remove excess surface water with constant manual pressure for 10 s and then weighed (m_{wet}). Then, it was dried in an oven at 30 °C to remove all the water until constant weight and determine its dry mass (m_{dry}). Then, equation 1, provided by Zhang et al.[30], was used to calculate the water retention capacity.

$$WRC = \frac{\left(m_{wet} - m_{dry}\right)}{m_{wet}} \times 100 \quad (1)$$

2.3.2 Rehydration percentage (RP)

BC membranes were characterized by the rehydration percentage (RP) according to the methodology described in Inoue et al.[31].

2.3.3 Dry mass yield

The dry mass yield was determined by weighting the membranes after drying (m_{dry}). Then, the average dry mass values obtained for all samples were normalized about KHGL, the standard cultivation medium. The Equation 2 was used to calculate the relative dry mass yield:

$$Relative\,dry\,mass\,yield\,(\%) = \frac{m_{dry\_sample}}{m_{dry\_KHGL}} \times 100 \quad (2)$$

Therefore, values greater than 100% mean that they had a dry mass yield higher than the standard (KHGL) and values lower than 100% the dry mass yield was lower than the standard. Quintuplicates were performed.

2.3.4 Thermogravimetric Analysis (TGA)

To verify the influence of culture media on the thermal stability of membranes. The samples were heated at a rate of 10 °C/min from room temperature to 1000 °C, under an inert atmosphere (N_2), in TA Instruments model TGA-Q50 equipment.

2.3.5 Fourier-Transform Infrared Spectroscopy (FTIR)

The characterization of the functional groups of the membranes was obtained by spectroscopy in the infrared region in equipment from Perkin Elmer Frontier. Scans were performed per sample, from 450 to 4000 cm^{-1}, with a resolution of 2 cm^{-1} in the Attenuated Total Reflectance (ATR) mode.

2.3.6 Analysis of Variance and Tukey's Test

Analyzes of variance (ANOVA) and Tukey's test were performed using Minitab 18 software to verify statistically significant differences between the different culture media.

3. Results and Discussions

Membranes were synthesized in flasks with 14 cm diameter, acquiring the same dimension. In Figure 1, there are images of the membranes produced, identified by a) KHMN (*K. hansenii* produced in mannitol), b) KHGL (*K. hansenii* produced in glucose), c) KHCP (*K. hansenii* produced in black tea and sucrose), and d) CMCP (microbial consortium produced in black tea).

Membranes produced in the black tea culture medium acquired a darker color than the other samples. According to Yim et al.[32], black tea contains polyphenol, which generates compounds that darken the membrane after its conversion, such as orange theaflavin. However, all membranes reached white color after purification.

The membranes were analyzed for dry and wet mass in their respective culture media, WRC and RP tests. The data obtained are presented as mean values and their respective standard deviations in Table 1.

The average dry mass values obtained for all samples were normalized about KHGL, the standard cultivation medium. This allowed us to compare the relative variation in the yield of bacterial cellulose produced by each condition. The results are shown in Figure 2.

It can be observed that the culture of *K. hansenii* containing mannitol (KHMN) obtained a dry mass yield of around half that obtained by the standard cultivate (KHGL). It is also observed that the yield presented by the same microorganism with sucrose as a carbon source (KHCP) was slightly higher, around 60% of the yield obtained by KHGL, which had glucose as a carbon source. However, it is essential to highlight that the crop that obtained the best yield was CMCP, presenting a yield of almost 350%, representing an increase in cellulose production of more than 3 times compared to the standard cultive.

According to Tureck et al.[33], glucose is readily transported across the cell membrane and incorporated into the cellulose biosynthetic pathway, causing more excellent film production using this carbon source. However, the culture medium containing commercial sugar (sucrose) does not occur in the same form. Sucrose must be hydrolyzed in the periplasm into glucose and fructose[34]. Therefore, this delay generates lower yield in the same incubation period. The same occurs with mannitol, which is first transformed into fructose to be later metabolized by the organism of the bacteria, producing cellulose[35]. Therefore, the lower yield of the carbon sources mentioned is related to the metabolism

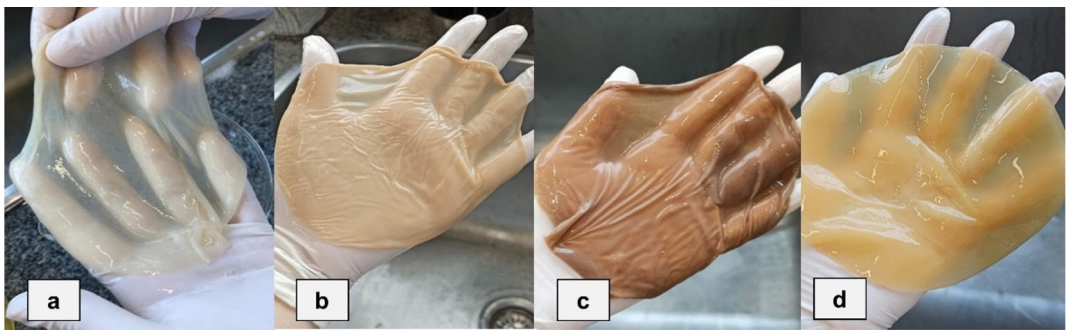

Figure 1. Membranes of BC synthesized before purification step (a) KHMN; (b) KHGL (c) KHCP; (d) CMCP.

Table 1. Dry mass, wet mass, water retention capacity (WRC) and rehydration percentage (RP) for KHMN, KHGL, KHCP and CMCP membranes.

Sample	Wet mass (g)	Dry mass (g)	WRC (%)	RP (%)
KHGL	2.779 ± 0.749	0.084 ± 0.005^{AB}	96.775 ± 0.732^{B}	28.404 ± 4.919^{A}
KHMN	2.115 ± 0.428	0.048 ± 0.022^{B}	97.856 ± 0.642^{AB}	20.714 ± 7.318^{AB}
KHCP	4.229 ± 0.929	0.056 ± 0.017^{B}	98.602 ± 0.469^{A}	15.735 ± 6.626^{BC}
CMCP	28.491 ± 13.752	0.289 ± 0.199^{A}	99.048 ± 0.240^{A}	5.564 ± 3.693^{C}

of their molecules. It is noteworthy that, compared to the mannitol and sucrose, there was a higher yield of membranes produced in a culture medium containing sucrose by the bacterium *K. hansenii* because it contains a higher carbon source concentration of 50 g/L.

Villarreal-Soto et al.[36] also explains that in the case of Kombucha, the different yeasts and bacteria species act in parallel, producing two different final products: the fermented tea and the biofilm. While yeasts convert sucrose into glucose and fructose, bacteria use these compounds already converted to produce cellulose. Through analysis of variance and Tukey test, the dry mass yield of the microbial consortium does not significantly differ from the yield obtained with *K. hansenii* in black tea medium. However, it is significantly different from the other experiments. There was no significant difference between the values of the culture media using mannitol and glucose as a carbon source and the black tea culture medium for *K. hansenii*.

Despite using different culture media and microorganisms, the WRC analysis demonstrates high water retention by all synthesized membranes, above 95%. Galdino et al.[37], using propolis extract and steep corn liquor, respectively, for synthesizing cellulose membranes as culture medium, obtained the same values for this parameter. Furthermore, according to Ullah et al.[38], highly porous BC favors a high-water holding capacity where the water molecules remain within the porous matrix. Rebelo et al.[39] explain that many hydrogen bonds in BC result in high water retention. The analysis of variance and Tukey test shows no significant difference between the samples produced by the microbial consortium (CMCP) and by *K. hansenii* in a black tea (KHCP) and culture medium containing mannitol as a carbon source (KHMN). The samples produced in a culture medium containing glucose (KHGL) do not significantly differ from those synthesized in a medium containing mannitol in its formulation (KHMN). However, KHGL is significantly different from the other samples.

It is important to highlight that the membranes formed decreases in thickness after drying in the oven when water is lost from its structure. The KHCP membrane, for example, decreased the thickness from 3.513 ± 0.049 mm to 0.019 ± 0.001 mm after drying (Figure S3, Supplementary Material).

As for the percentage of rehydration (RP) (Table 1), it was identified through analysis of variance and Tukey test that there is no significant difference between culture medium containing glucose (KHGL) and mannitol for *K. hansenii* (KHMN), mannitol (KHMN) and black tea for *K. hansenii* (KHCP), and black tea for *K. hansenii* (KHCP) and the microbial consortium (CMCP). Therefore, there was a significant difference between the membranes, indicating that those produced by *K. hansenii* can reabsorb a more significant amount of water than the films produced by SCOBY. The interaction between the cellulose matrix and the water molecules also changes according to the arrangement of the cellulose fibers; the more relaxed the interaction, the more efficient it is. Fibers from samples produced by the microbial consortium may find themselves more tightly interconnected, resulting in less space to re-accommodate water. Illa et al.[40] explain that although the cellulose formed has a high water retention capacity, water needs to be removed for most of its applications, thus using several techniques that affect the properties of the membrane.

Considering that the membranes produced were oven-dried, Inoue et al.[31] reports that the cellulose fibers collapse due to the rapid evaporation of water, making the water molecules, when rehydrating them, unable to overcome the intermolecular forces formed and go back to the original structure. Lin et al.[41] justifies the low rehydration of dry membranes due to the high crystallinity of membranes, also affecting their permeability. According to Leonarski et al.[42], the more porous surface of the formed membrane remains in contact with the liquid, while the more crystalline structure is in contact with the air. Therefore, when analyzing the membranes formed by the microbial consortium, there is an abundant growth of the film above the surface of the liquid, which makes the more crystalline part (in contact with air) more significant than the porous part of the membrane, also justifying the low rehydration of this membrane.

In order to assess possible changes in the chemical structures of the cellulose membranes synthesized as a function of the change in the carbon source, the FTIR analysis was performed. Figure 3 shows the results for the samples. In all samples, bands related to BC were identified, such as stretching (ν) of the hydroxyl groups at 3340 cm^{-1}, C-H and CH_2 stretching at 2890 cm^{-1}, symmetrical angular bending (δs) of H-O-H at 1626 cm^{-1}, symmetrical angular bending of C-OH and CH at 1428 and 1313 cm^{-1}, stretching of the C-O at 1180 and 983 cm^{-1}, and C-H angular bending (δ) at 896 cm^{-1}[43]. It is

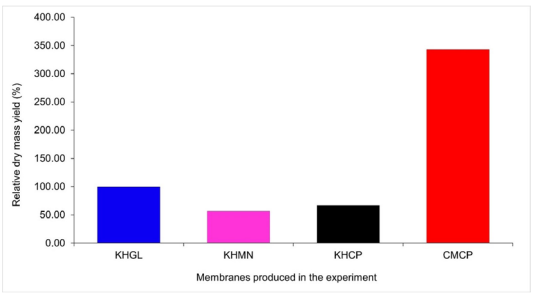

Figure 2. Yield of membranes in dry mass.

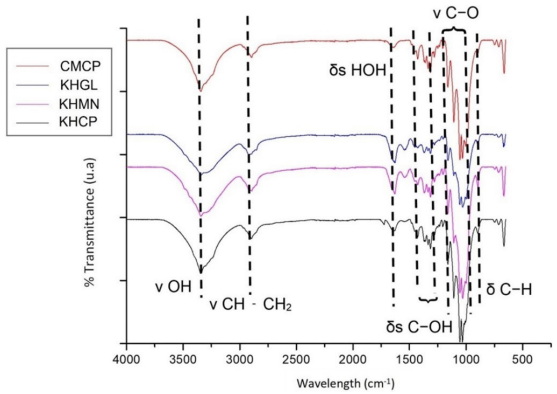

Figure 3. FTIR analysis of KHMN, KHGL, KHCP and CMCP samples and characteristic binding of bacterial celulose.

possible to observe that all samples show the same behavior. Therefore, BC was also obtained as a product with similar chemical structures when using the microbial consortium and other culture media.

From the thermogravimetric analysis (TGA) (Figure S4, Supplementary Material), information about the maximum thermal degradation temperature (T_{peak}) and mass loss of the membranes were extracted to form Table 2.

The first thermal event, ranging from approximately 30 °C to 250 °C, indicates the loss of mass related to the evaporation of residual water from the drying process. Already in the second stage (325 to 357 °C), the samples suffer a marked mass loss (about 68%), corresponding to cellulose degradation (dehydration and decomposition of glycosidic units). The third and last stage, around 430 °C, is related to the thermoxidative degradation of cellulose.

The CMCP sample obtained a higher temperature during the second thermal event referent cellulose degradation (T_{peak2} 285 °C) (Table 2 and Figure S4, Supplementary Material), while the KHGL sample (standard cultivation medium) presented T_{peak2} of 256 °C. This result suggests that the BC obtained from the symbiotic association pointed out to have more thermal stability than that produced from only one bacterial species. One possibility is the high density of fiber and arranged more firmly linked. With most of the membrane composed of a crystalline network, the temperature required for its degradation has increased. The temperatures and loss mass values obtained by CMCP were near values found by Avcioglu et al.[44] using black tea and glucose in membrane production.

The KHMN sample showed the lowest maximum temperatures in all stages. Studies conducted by Mohammadkazemi et al.[45] demonstrated that different carbon sources could result in different morphologies, resulting in properties such as crystallinity and orientation of different fibers. These structural parameters affect the behavior of thermal degradation. For this reason, the samples KHMN, KHCP and KHGL, although the same microorganism synthesized them, showed degradation behavior in the three stages and different waste content due to the different carbon sources used in cultivation.

According to Molina-Ramírez et al.[46], the initial degradation temperature of BC occurs between 220-300 °C, and its maximum degradation temperature is reported between 348-361 °C. Therefore, when comparing this information with Table 2, it was concluded that all sample temperatures are within the cited ranges. The temperatures were compared with those obtained by other authors, represented in Table 3. It is worth highlighting that the temperature variations between them are a function of the microorganism and the culture medium.

In addition to the more extensive cellulose production, the characterization analyses demonstrated that the material produced from the pure strain and microbial consortium is the same. It is essential to highlight that the microbial consortium achieved this result using a lower-cost alternative medium without temperature control and sterilization of the medium and the culture vessel, reducing the membrane's production cost. In the fermentation process, the cost of the medium is responsible for 50-65% of the total expense[20]. In this way, a price survey was carried out, and other waste used by other authors was considered for comparison with the results obtained in this study (Table 4).

Thus, to prepare 1 liter of culture medium for the formation of the BC membrane, there was a 92.65% cost reduction considering only the medium. The HS standard medium totals USD 1.36/L, while the value per liter of the alternative culture medium was just USD 0.10/L. In a study, Avcioglu et al.[44] found a reduction of almost 30% in the cost of the culture medium using the alternative medium for kombucha synthesis instead of the synthetic medium. However, such a difference in cost reduction compared to the present work is justified by using glucose as a carbon source. At the same time, the author evaluated using mannitol as a carbon source. Considering all of the options of culture media using waste materials to produce bacterial cellulose, the black tea used in this study is still an alternative to reduce the cost of production of BC. One of the advantages of using the microbial consortium, according to Villarreal-Soto et al.[36], is the possibility of producing cellulose from various carbon sources, including glucose, ethanol, sucrose and glycerol. As a result, the kombucha

Table 2. Maximum thermal degradation temperature (T_{peak}) and mass loss data for BC membranes obtained from thermograms.

Sample	Mass loss 1 (%)	T_{peak1} (°C)	Mass loss 2 (%)	T_{peak2} (°C)	Mass loss 3 (%)	T_{peak3} (°C)
KHMN	5.2	242	67.5	325	9.5	421
KHCP	6.2	262	69.3	349	5.5	428
KHGL	5.1	256	68.8	345	6.9	428
CMCP	6.6	285	69.3	357	-	-

Table 3. Comparison of temperature ranges found by other authors concerning TGA and DTG.

Reference	Microorganism used	Culture medium	T_{onset} (°C)	T_{peak} (°C)
Costa et al. [23]	*Gluconacetobacter hansenii*	HS modified with corn steep liquor	265	310
Molina-Ramírez et al.[46]	*Komagataeibacter medellinensis*	Rotten mango juice	240	327
Avcioglu et al.[44]	*Kombucha microbial consortium*	lack tea and glucose	250	350
Gündüz and Aşık[47]	*Gluconacetobacter hansenii*	Carrot Juice	259	335

Table 4. Comparison with the literature of Hestrin-Schramm culture medium and black tea in the production of microbial cellulose in terms of cost reduction (%).

Reference	Culture media	Reagents	Total price (USD/L)
This study	Black tea	Black tea, commercial sugar	0.10
This study	Standard HS	Glucose, peptone, yeast extract, sodium phosphate bibasic, citric acid	1.36
Amorim et al.[48]	Fruit residue juice	Fruit residue juice	0.94
Zhou et al.[49]	Supplemented jasmine flower, enzymatic hydrolyzate	Jasmine flower enzymatic hydrolyzate, yeast extract, tryptone	3.29
Liu et al.[50]	Vinegar residue	AC treated vinegar residue, sugar, lignin	5.58
Souza et al.[51]	Cashew apple juice	Cashew apple juice, crude soybean molasses	1.34

industry has overgrown in recent years, and research related to kombucha is flourishing[52]. In other words, the greater the growth of this segment in the market, the greater the production of BC can also become. However, one of the challenges to be overcome is the standardization of the composition of these microorganisms in the microbial consortium to establish a uniform and controlled production.

4. Conclusions

All experiments performed synthesized BC membranes, both as well *K. hansen*ii as the association of fungi and bacteria (SCOBY) in different culture media, with a higher yield in dry mass for membranes produced by the microbial consortium in the same period of growth than the isolated bacterium. The characterization of the membranes showed that they were of the same composition, showing, however, better thermal stability than membranes formed by *K. hansenii*. Notably, the film formed by the association of microorganisms, in addition to having been synthesized in the lowest cost medium (black tea and sucrose), did not require temperature control (room temperature) and sterilization of the culture medium. Thus, it is concluded that it is possible to obtain BC membranes from the association of microorganisms (SCOBY), using lower-cost culture media and presenting the same properties as those produced by the pure strain in a synthetic culture medium, allowing its large-scale production.

5. Author's Contribution

• **Conceptualization** – Izabel Cristina Nóvak; Andréa Lima dos Santos Schneider.

• **Data curation** – Izabel Cristina Nóvak.

• **Formal analysis** – Izabel Cristina Nóvak.

• **Funding acquisition** - Andréa Lima dos Santos Schneider.

• **Investigation** – Izabel Cristina Nóvak; Bruna Segat.

• **Methodology** – Izabel Cristina Nóvak; Andréa Lima dos Santos Schneider.

• **Project administration** – Andréa Lima dos Santos Schneider.

• **Resources** – Michele Cristina Formolo Garcia; Ana Paula Testa Pezzin; Andréa Lima dos Santos Schneider.

• **Software** – NA.

• **Supervision** – Andréa Lima dos Santos Schneider.

• **Validation** – Izabel Cristina Nóvak; Michele Cristina Formolo Garcia; Andréa Lima dos Santos Schneider.

• **Visualization** – Izabel Cristina Nóvak.

• **Writing – original draft** – Izabel Cristina Nóvak.

• **Writing – review & editing** – Bruna Segat; Michele Cristina Formolo Garcia; Ana Paula Testa Pezzin; Andréa Lima dos Santos Schneider.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. Process of synthesis of bacterial cellulose membrane from Komagataeibacter hansenii ATCC 23769.

Figure S2. Process of bacterial cellulose membrane synthesis from a microbial consortium.

Figure S3. KHCP membrane a) before and b) after oven drying.

Figure S4. TGA analysis of samples: a) TG curves; b) DTG curves.

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