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Propolis: as an Additive in Bacterial Cellulose Production

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Abstract

This study investigates the effect of propolis supplementation on bacterial cellulose (BC) production efficiency with *Komagataeibacter* species. Compared to production in Hestrin-Schramm medium, the addition of propolis increased BC production with *K. intermedius*, *K. maltaceti*, and *K. nataicola* by 1.31-fold, 2.09-fold, and 1.43-fold, and optimal propolis concentration were determined to be 25%, 20%, and 30%, yielding 7.15 g/L BC, 5.4 g/L BC, and 4.15 g/L BC, respectively. *K. intermedius* - *K. maltaceti* consortia, increased production by 1.57-fold compared to *K. intermedius* and 2.07-fold compared to *K. maltaceti* monocultures. Increasing the volume of the cultivation vessel also increased BC production by 1.08-1.59-fold. Agitation induced production efficiency by 1.01-1.18-fold; however, obtained BC exhibited irregular shapes. BC obtained from *K. maltaceti* exhibited the highest Water Holding Capacity (WHC) and Moisture Content Retention (MCR) as 97.63% and 33.22 g/g. Characteristic BC bands and nanofibrillar structure of BC were observed with Focused Ion Beam Scanning Electron Microscopy (FIB-SEM) and Fouirer Transform Infrared (FT-IR) Spectrometer.

Keywords: Bacterial cellulose, Bacterial consortia, Propolis, *Komagataeibacter* sp.

1. Introduction

Propolis is a traditional therapeutic agent collected by bees from plant secretions, containing active compounds such as soluble polyphenols and flavonoids in its structure. Besides exhibiting antimicrobial properties, showing protective effects against bacteria and fungi in bee hives, it is also known to possess antiviral and antiparasitic effects [1-4]. Propolis also contains various amino acids, minerals, sugars, and many other valuable components and can be used as an *ex situ* and *in situ* agent in the modification of polymers due to its unique properties [4-8].

Bacterial cellulose (BC) is a carbohydrate-based biopolymer synthesized extracellularly by microorganisms, forming a hydrogel membrane on the culture surface [5, 8]. With its ultrapure structure, high water retention capacity, biocompatibility, porosity, mechanical strength, high crystallinity, large surface area, and biodegradability, BC is increasingly being used in industrial areas, including food, biomedicine, cosmetics, textiles, and acoustics [5,9-11]. The fibrous structure of BC, formed by hydrogen bonds, creates a dense polymer matrix that is insoluble in water and organic solvents, making it a preferred material [12]. The easily controllable structure and being obtained in a pure form from the cultivation media makes this biomaterial a significant advantage for its preference in industrial applications [13].

The high cost of the cultivation medium used in BC production and the low production yield limit its industrial use. Therefore, it is crucial to use low-cost substrates and additives in Hestrin Schramm (HS) broth or replace the ingredients in HS medium to obtain BC with a high yield [13-16]. Increasing industrial production, reducing costs, and improving the mechanical properties of BC are crucial for enhancing product yield [10]. Additionally, the properties and morphology of the BC obtained during production are directly influenced by the nutrients comprising the culture medium and cultivation conditions such as pH, oxygen, and temperature. Therefore, modifying structural and physiological properties can be achieved by using additives during BC fermentation [17]. Through *in situ* modifications, it becomes possible to alter the

properties of BC fibrils such as size, surface area, crystallinity, electrical conductivity, and mechanical characteristics [13,18]. Despite lacking antimicrobial properties, due to its easily moldable structure and the ability to integrate with various substances and release the integrated material, this biopolymer is commonly modified with the use of additives during the fermentation process or combined with additives to create composites through *ex situ* modification [5, 16, 19, 20]. Accordingly, carboxymethylcellulose (CMC) [21], gellan gum [13], silicone polyether surfactant (SPS) [16], pullulan [10], ethanol and lactic acid [22], and polyethylene terephthalate ammonia hydrolysate (PETAH) [23] used as additives in BC production and resulting as to highly form product yield in the literature.

Indeed, in addition to *in situ* and *ex situ* modifications, it is possible to modify BC with co-cultivation by using multiple microorganisms in the fermentation media [20]. Co-cultivation is noted to be a method that reduces production costs in biotechnological applications and alleviates metabolic burdens among microorganisms through factors such as cross-feeding of nutrients when microbial consortia are used together [19, 24]. In recent years, an increasing trend in co-cultivation has been observed in BC production with the use of *Komagataeibacter* sp. and *Lactocaseibacillus* [20], *K. sucrofermentans*, *Leuconostoc mesenteroides* and *Xanthomonas campestris* [25], *Saccharomyces cerevisiae* and *K. rhaeticus* [11], and *Enterobacter* sp. and *Lactobacillus lactis* [19].

In this study, propolis was used as an additive in HS broth to determine the effect of propolis on BC production with *Komagataeibacter* species (*K. intermedius, K. maltaceti* and *K. nataicola*) and their consortia (*K. nataicola - K. intermedius, K. maltaceti - K. nataicola, K. intermedius - K. maltaceti*). Additionally, inoculum ratios of binary consortia of *Komagataeibacter* species and the impact of culture volume (2 mL, 10 mL, and 20 mL with the vessel dimensions of 15mm, 45mm and 85mm, respectively) on BC production was investigated. The effect of agitation on BC yield was also evaluated. Furthermore, obtained BC membranes were characterized using Focused Ion Beam Scanning Electron Microscopy (FIB-SEM) and Fourier Transform Infrared (FT-IR) Spectrometer, and Water Holding Capacity (WHC) and Moisture Content Ratio (MCR) were also calculated, which are crucial data for the industrial use of this valuable biomateral.

2. Materials and Methods

2.1. *Komagataeibacter* strains and pre-cultivation conditions

K. intermedius, K. maltaceti and K. nataicola strains obtained from Avcioglu's research [14, 15, 26] were used as BC producers in this study. Pre-cultivation was performed in HS broth (containing in g/L: 20 glucose, 5 peptone, 5 yeast extract, 2.7 Na_2HPO_4 and 1.15 citric acid) at 30˚ C for 3 days statically (MCI 120, Mipro, Ankara, Turkey). Cultures with fibril formation were used as pre-culture in the rest of the study [26].

2.2. Propolis effect on BC production

Propolis, obtained from a local manufacturer (Ankara, Turkey), was used as an additive in HS broth to investigate the effect on BC yield by using *K. intermedius, K. maltaceti* and *K. nataicola* species. Accordingly, 5-40% concentrations of propolis were added in HS broth and 10% (v/v) of each bacterial culture (OD_{600nm} = 0.20-0.25) were inoculated, separately. Optimal propolis concentration for each *Komagataeibacter* species was evaluated after an incubation period at 30˚ C for 7 days in a static incubator (MCI 120, Mipro, Ankara, Turkey). HS broth without propolis addition was used as a negative control for demonstrating the difference between production yield incubated in propolis included media [26].

2.2.1. Effect of incubation period on BC production

To compare BC production, pH and bacterial growth (OD_{600nm}) during the incubation period (1-7 days) with *Komagataeibacter* species, incubation was performed at 30˚ C in a static incubator (MCI 120, Mipro, Ankara, Turkey) and all parameters were evaluated for each day from 1 to 7.

2.2.2. BC production with *Komagataeibacter* consortia

To investigate the effect of propolis supplementation to HS broth on BC production with binary consortia of *Komagataeibacter* species (*K. intermedius-K. nataicola, K. maltaceti-K. intermedius* and *K. nataicola-K. maltaceti*), 1:1, 1:2, 1:4 and 1:8 inoculum ratio of *Komagataeibacter* species were inoculated in propolis including HS broth, separately. Incubation was performed at 30˚ C for 7 days in a static incubator (MCI 120, Mipro, Ankara, Turkey).

2.2.3. Effect of cultivation volume/dimension and agitation on BC formation

Komagataeibacter species were inoculated in propolis including HS broth with a production volume of 2 mL, 10 mL and 20 mL (and vessel dimensions of 15mm, 45mm and 85mm, respectively). Incubation was performed at 30˚ C for 7 days in a static incubator (MCI 120, Mipro, Ankara, Turkey). Cultures also incubated in agitated condition at 150 rpm, 30˚ C for 7 days (MCI 120, Mipro, Ankara, Turkey). Obtained BC membranes were compared using BC yield and membrane form.

2.3. BC purification

Harvested BC membranes from the surface of the cultivation media were treated with 0.1 M NaOH

solution at 80 °C for 30 min and entrapped cells were removed by washing distilled water until reaching neutral pH. Purified membranes were dried at 30 °C and the dry weight of BC was calculated [14, 15].

2.4. Moisture content ratio (MCR) and Water holding capacity (WHC) of BC membranes

The weight of the wet and dried BC membranes was used to calculate the moisture content ratio (MCR) of the membranes as follows;

$$\text{MCR (\%)} = \frac{\text{Wwet}-\text{Wdry}}{\text{Wwet}} \text{ x } 100 \quad (\mathbf{2.1}) \qquad [27]$$

$$\text{WHC (g/g)} = \frac{\text{Wwet}-\text{W dry}}{\text{Wdry}} \qquad (\mathbf{2.2}) \qquad [14]$$

2.5. Characterization of BC membranes

Morphological and dimensional characteristics of purified BC membranes obtained from propolis including media were analyzed with FIB-SEM GAIA3, Tescan, operating at 3 kV with a magnification of 60 kx [15, 26]. Additionally, FT-IR spectra of BC membranes were collected on the Vertex FT-IR (Bruker, Germany) over the range of 4000 to 400 cm^{-1} with a resolution of 1 cm^{-1} and 20 scans in the region.

2.6. Statistical analysis

SPSS (The Statistical Package for the Social Sciences) 23 program (Chicago, IL, USA) was used to evaluate the obtained data. The mean, standard deviation and median are given, and the results of BC production with propolis supplementation were compared using the Kruskal-Wallis H test with a 5% significance level.

3. Results and Discussion

3.1. Propolis effect on BC production

Due to its nanofibrillar structure and the unique properties such as high water-holding capacity, mechanical strength, porosity, and ease of modification, BC has gained significant interest from researchers in recent years. For this reason, studies to improve its production and structural properties are actively continuing [28, 29]. The presence of free hydroxyl groups on the membrane surface allows BC to be modified with other polymers or additives [30]. Therefore, increasing the production efficiency of this valuable biopolymer and improving its existing mechanical properties is only possible by supplementing it with additives to the cultivation media or modifying it with various agents following the production process [8, 15].

Accordingly, current methods to improve the production yield of BC include the supplementation of additives to commercial media or the substitution of these media with low-cost substrates. In this study, the effect of propolis supplementation, which has rich nutritional content, at different concentrations (5-40%) to HS medium on BC production with *Komagataeibacte*r species was investigated. The optimal propolis concentrations for *K. intermedius*, *K. maltaceti*, and *K. nataicola* were determined to be 25%, 20%, and 30%, respectively, yielding 7.15 g/L BC (20.09 g/g WHC, 96.31% MCR) ($p=0.002$, Table S1a), 5.4 g/L BC (31.01 g/g WHC, 96.87% MCR) ($p=0.002$, Table S1b), and 4.15 g/L BC (21.67 g/g WHC, 95.59% MCR) ($p=0.002$, Table S1c). It was observed that the effect of the propolis concentration on BC yield varied depending on the species, and there was a decrease in BC production at concentrations above the optimal value (**Figures 1a, 1b and 1c**). Compared to the propolis-free medium (HS broth) used as a negative control, *K. intermedius*, *K. maltaceti*, and *K. nataicola* strains were found to cause an increase in production efficiency as 1.31-fold (5.47g/L), 2.09-fold (2.58g/L), and 1.43-fold (2.91), respectively (data not shown). Therefore, in addition to the use of the nutritional elements contained in HS, it was observed that the propolis content was also used as carbon and nitrogen sources and contributed to the production of BC up to a certain concentration. However, it was concluded that as the amount of propolis, which contains essential oils, resins, polyenes, and various organic and inorganic chemicals, as well as flavonoids and phenolics, increases in the medium, it also acts as an antimicrobial agent in the cultivation media [31-33]. Thus, Avcioglu 2024 [15] found that the total phenolics in the plant content contributed to BC production at optimal plant concentrations. Therefore, the inclusion of propolis content, in addition to the HS medium, contributes to BC production. Amorim et al. 2022, [5] was found that propolis supplementation to the production medium of *G. hansenii* prevented reproduction and suppressed BC production even in the medium containing 25% propolis [5]. However, the data obtained from this study show that the addition of propolis, which has a rich content, has a positive effect on BC production efficiency up to certain concentrations, and that increasing the amount of propolis reduces BC production as stated by Amorim et al. 2022 [5]. Propolis, which has antimicrobial properties, was interpreted as a decrease in BC production by inhibiting bacterial growth. Therefore, by taking advantage of the antimicrobial effect of propolis, red propolis is used for wound healing in diabetic patients with bacterial cellulose membrane [34], propolis included chitosan-based films used for food packaging [35].

In the literature, many additives other than propolis have been used in BC production and have been found to significantly contribute to BC yield. Accordingly, the use of carboxymethylcellulose (CMC) in the cultivation media increases BC production from 1.3 g/L to 8.2 g/L [21], and that gellan gum used as an additive in BC production increases the production efficiency by 47-59% [13]. In a different study, it was stated that the

addition of konjac glucomannan and xanthan gum increased the BC production efficiency resulting in 6.97 g/L and 6.52g/L, respectively [31]. The effect of ethanol and lactic acid on BC production by *Gluconacetobacter kombuchae* was determined that these supplements had an increasing effect on BC production efficiency compared to HS. Also, it was found that the use of 0.6% lactic acid caused as 4.89 g/L BC, and the use of 1% ethanol caused as 3.7 g/L BC production [22]. HS broth enriched with 1% polyethylene terephthalate ammonia hydrolysate (PETAH) caused a 215% increase in production efficiency in BC production with *Taonella mepensis*, a Gram-negative bacterium [23]. Accordingly, this study concluded that the use of propolis as an additive in BC production caused an increase in BC production efficiency, but increased concentrations caused a decrease in BC efficiency as it suppressed its nutritional quality and made its antimicrobial properties dominant.

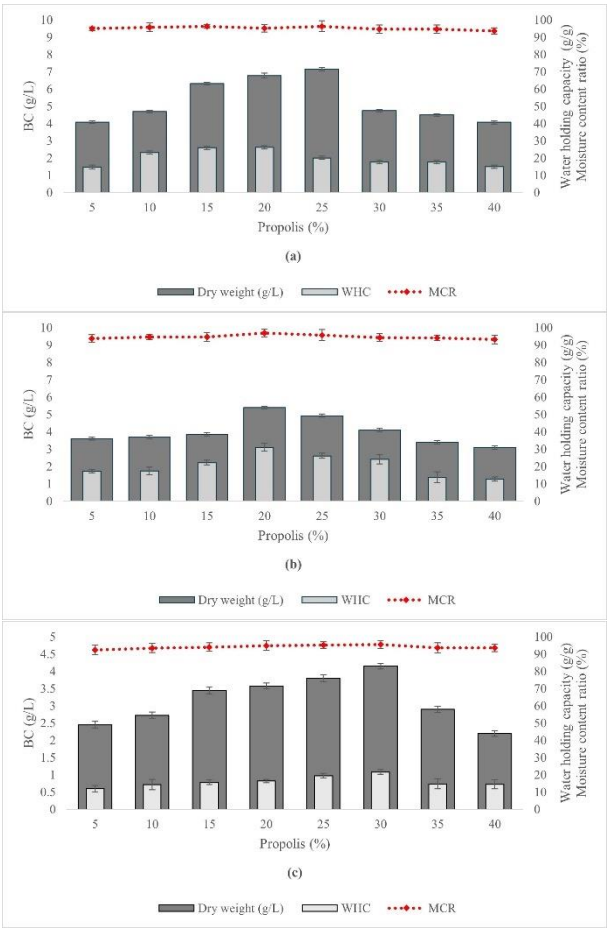


Figure 1. Effect of propolis supplementation on BC production with (a) *K. intermedius*, (b) *K. maltaceti*, (c) *K. nataicola.*

3.2. Effect of incubation period on BC production

BC formation increases with the growth of bacteria inoculated into the fermentation medium and the increase in C-H bonds between the BC fibrils formed. However, as pellicle formation slows down and all bacteria in the culture medium are trapped in the formed pellicle, BC synthesis reaches the production threshold [36]. Additionally, where time-dependent production continues and nutrients are not added regularly to the medium, it is inevitable that reproduction and biopolymer synthesis will stop because of the consumption of nutrients by the growing microorganisms. In this study, it was determined that pellicle formation in all three *Komagataeibacter* species started from the 3rd day and there was a decrease in the pH of the culture liquid due to bacterial growth resulting as formation of acidic products (**Figure 2a, 2b and 2c**). It is known that the rapid decrease in pH is mainly due to the presence of organic acids, especially gluconic acid, formed in the culture medium with the growth of acetic acid bacteria [37]. This is a result of the glucose contained in the medium being rapidly used by bacteria in the reproductive stage, causing gluconic acid accumulation [38]. In this study, as a result of bacterial growth, the culture pH of *K. intermedius*, *K. maltaceti* and *K. nataicola* decreased from 6.0 to 2.93 (p=0.003, Table S2a), 3.11 (p=0.003, Table S2b) to 3.36 (p= 0.004, Table S2c), respectively. In addition, the pH of the culture media, bacterial growth and the amount of BC production during the BC production process varied species-specifically (**Figure 2**).

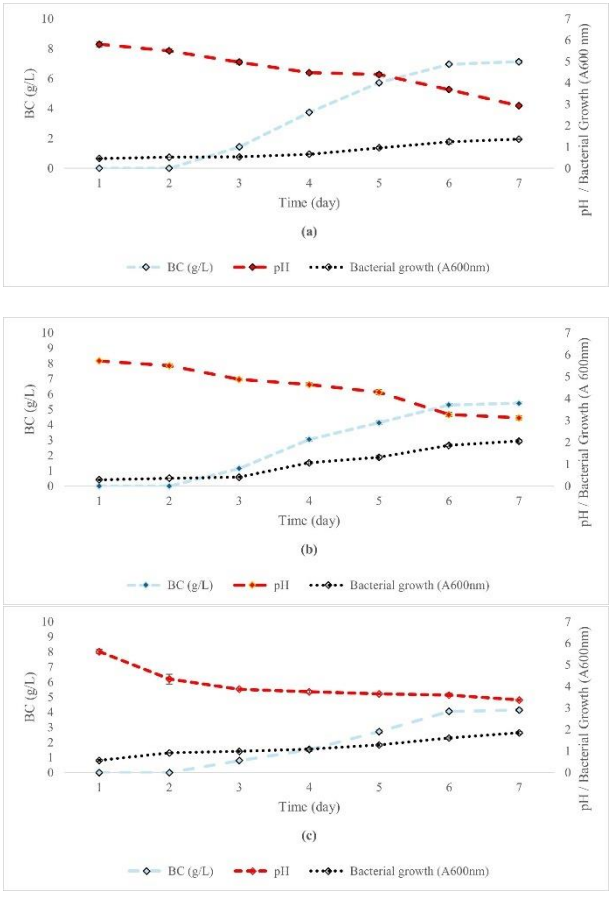


Figure 2. The change in the bacterial growth, pH and BC production during incubation period of **(a)** *K. intermedius*, **(b)** *K. maltaceti*, **(c)** *K. nataicola*.

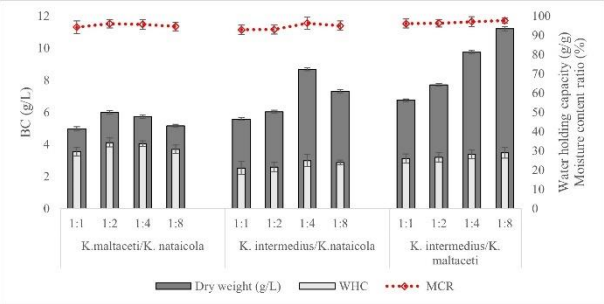


Figure 3. Effect of co-cultivation of *Komagataeibacter* species on BC production

3.3. BC production with *Komagataeibacter* consortia

Studies have shown that the co-cultivation of microorganisms causes an increase in biopolymer production and supports the development of the physicochemical properties of the resulting biopolymers [11, 19, 20]. In the literature, the co-culture of acetic acid and lactic acid bacteria causes the formation of BC-Hyaluronic Acid composites that can be used in biomedical and cosmetic fields [20]. In a different study, nisin-containing BC production was determined by *Enterobacter* sp. FY-07 and *Lactococcus lactis* N8 bacteria resulted in the synthesis of BC film with antimicrobial properties [19] and BC production was carried out using a co-culture of *Saccharomyces cerevisiae* yeast and *Komagataeibacter rhaeticus* bacteria [11]. Also, BC production efficiency increased from 2.64 to 5.99 g/L with the co-culture of *Komagataeibacter sucrofermentans* and *Leuconostoc mesenteroides* [25]. Accordingly, it was found that 5.99 g/L BC was produced (34.25 g/g WHC, 95.98% MCR) with 1:2 co-culture of *K. maltaceti-K. nataicola* (p=0.019, Table S3a), 8.67 g/L BC was obtained (24.92 g/g WHC, 96.27% MCR) (p=0.016, Table S3b) with 1:4 co-culture of *K. intermedius-K. nataicola*, and 11.20 g/L BC was produced (28.19 g/g WHC, 96.27% MCR) (p=0.016, Table S3c) with 1:8 co-culture of *K. intermedius-K. maltaceti* in this study (**Figure 3**). Additionally, *K. intermedius-K. maltaceti* consortium, which achieved the highest yield, increased production by 1.57-fold compared to *K. intermedius* and 2.07-fold compared to *K. maltaceti* monocultures. Therefore, the contribution of co-cultivation to biopolymer yield was supported across all consortia investigated in this research.

3.4. Effect of cultivation volume/dimension and agitation on BC formation

BC is a unique biopolymer that takes the shape of the fermentation vessel where it occurs, thus allowing it to be obtained in the desired size and shape [30,39]. When the effect of changing production volume and container diameter on production efficiency was examined, it was found that increasing the production volume of *K. intermedius* by 5-fold resulted in a 1.24-fold increase in BC yield (8.86 g/L), and a 10-fold increase in production volume (20 mL with a vessel dimension of 85 mm) led to a 1.30-fold increase in production efficiency (9.73 g/L) (p=0.016, Table S4a). Similarly, a 5-fold increase in volume resulted in a 1.08-fold increase in BC yield (5.86 g/L), and a 10-fold increase in volume led to a 1.27-fold increase in production efficiency (6.82 g/L) (p=0.022, Table S4b) with *K. maltaceti*. The respective increases were 1.38-fold (5.72 g/L) and 1.59-fold (6.57 g/L) with a 5-fold and 10-fold increase in volume (p=0.016, Table S4c) with *K. nataicola* (**Figures 4a, 4b, 4c and Figure 5**). Therefore, it was found that production volume had an inducing effect on BC production efficiency, depending on the increase in the amount of nutrients in the culture media. Similarly, it has been found in the literature that the increase in fermentation volume increases the formation of BC pellicle and thus BC production efficiency, as it provides high amounts of nutrients for bacterial growth [39].

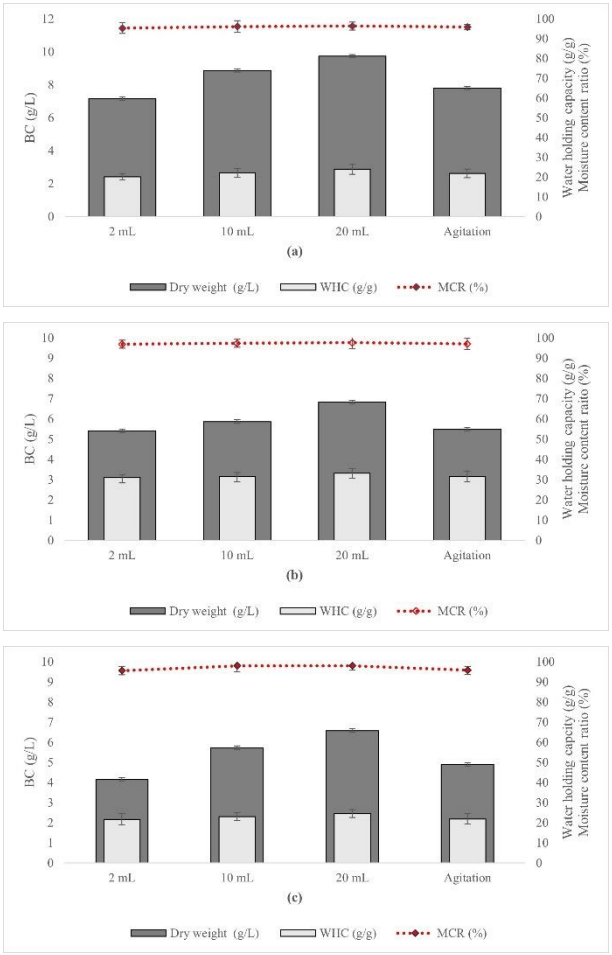


Figure 4. Effect of cultivation volume/dimension and agitation on BC production with (a) *K. intermedius*, (b) *K. maltaceti* and (c*) K. nataicola*.

Static cultivation requires a long cultivation time and extensive steps for BC production, resulting in a

gelatinous BC pellicle on the air surface of the culture medium [40]. However, it is seen that BC formation with agitation occurs in a short time and is widely used in commercial fermentative production [41]. While a layer-shaped BC membrane is obtained with static cultivation, irregular pellets, irregular masses or fibrous granules are formed in agitated conditions [41-43]. In this study, agitated production caused an increase in BC production of 1.08-fold (p=0.016, Table S4a), 1.01-fold (p=0.022, Table S4b) and 1.18-fold (p=0.016, Table S4c) compared to the static production of *K. intermedius*, *K. maltaceti* and *K. nataicola*, respectively. However, similar to the literature, it was observed that BC produced as a membrane under static production lost its layer form under agitated conditions and irregular BC formation was observed. Although it is very important to obtain products in a short time in biotechnological production processes, the importance of static culture is obvious since the materials whose surface properties need to be used are biopolymers produced in membrane form and are more suitable for modification for industrial use.

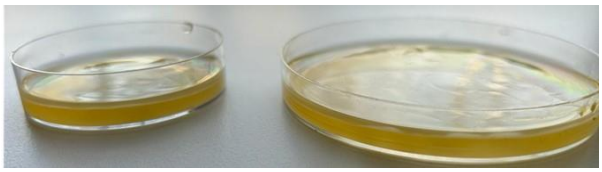
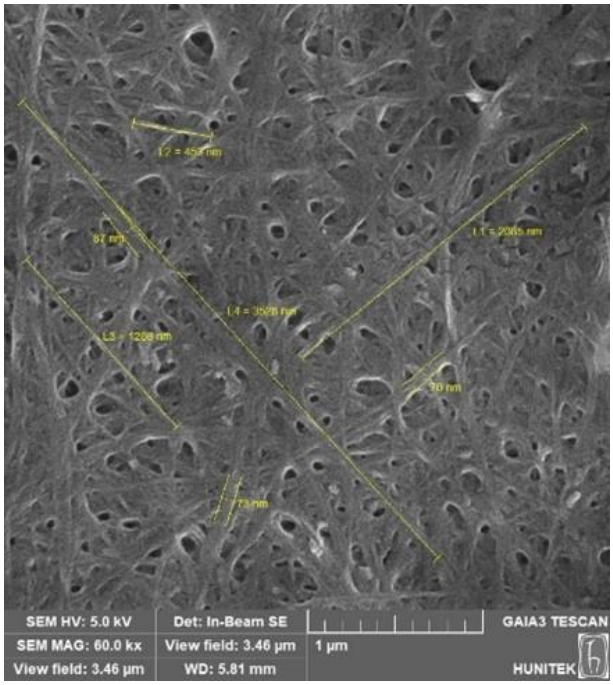
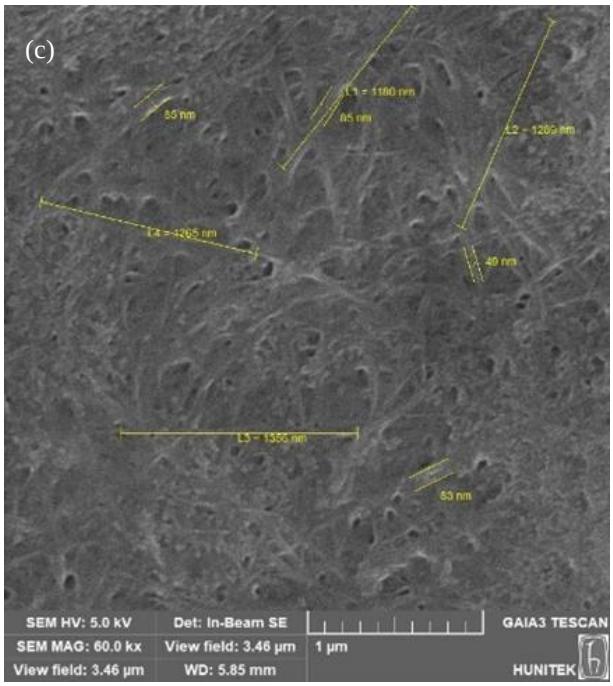


Figure 5. Images of BC membranes produced in different cultivation volumes/dimensions.

3.6. Characterization of BC membranes
3.6.1. FIB-SEM

When FIB-SEM images of obtained BCs were examined, it was determined that BC membranes exhibited 3-D, porous and nanofibrillar structure similar to the literature [14, 15, 26]. *K. intermedius*, *K. maltaceti* and *K. nataicola* species show fibrillar structure with widths of 51-59 nm, 49-85 nm and 70-87 nm, and lengths of 1502-2668 nm, 1180-1356 nm and 435-2085 nm, respectively (**Figure 6**).
BC membranes are known to have a water-holding capacity (WHC) of approximately 100 times their own weight [24]. It was found that *K. maltaceti* exhibited the highest WHC and Moisture Content Retention (MCR) values, ranging from 96.87% to 97.63% and 30.01 g/g to 33.22 g/g, respectively. This high capacity was correlated with its fibril size, which measured around 45 nm (**Figure 4 and Figure 6**). Therefore, in this study where propolis was used as an additive, it was observed that BCs with high WHC and MCR ratios were obtained.





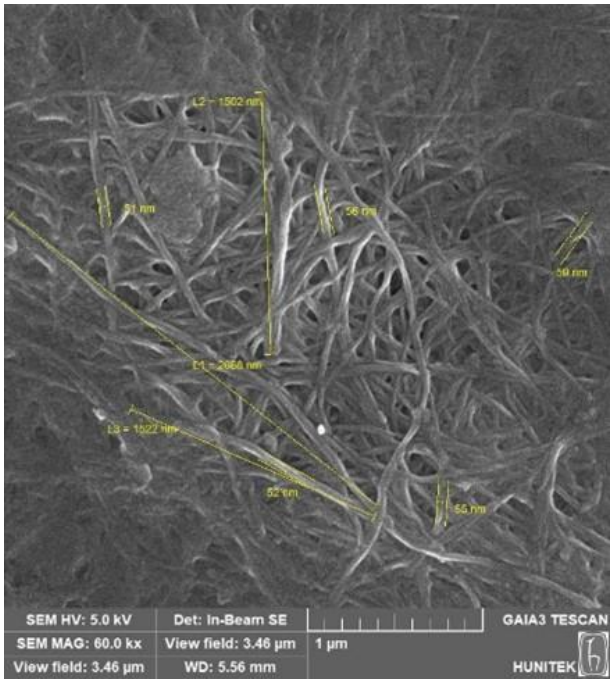
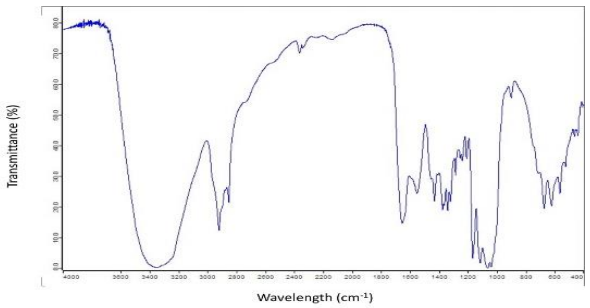


Figure 6. FIB-SEM image of BC fibrils obtained from **(a)** *K. intermedius*, **(b)** *K. maltaceti* and **(c)** *K. nataicola*.

3.6.2. FTIR analyzes

As a result of FTIR obtained from *K. intermedius*, *K. maltaceti* and *K. nataicola* species, it was determined that the membranes of each species showed characteristic vibration peaks. Accordingly, 3000-3600 cm^{-1} shows the hydrogen-bonding region and mainly corresponds to -OH stretching vibration, 2800-3000 cm^{-1} refers to C-H stretching vibration, the bands at ~1600 cm^{-1} corresponds to O-H bonds in adsorbed H_2O molecules, the bands at ~1300-1400 cm^{-1} refers to C-H and CH_2 bending and 900 ~1200 cm^{-1} refers to the stretching vibrations of carbohydrate molecules mainly corresponds to C–O–C, C–OH, C–H stretching vibration [15, 28, 44-46] (**Figure 7**).



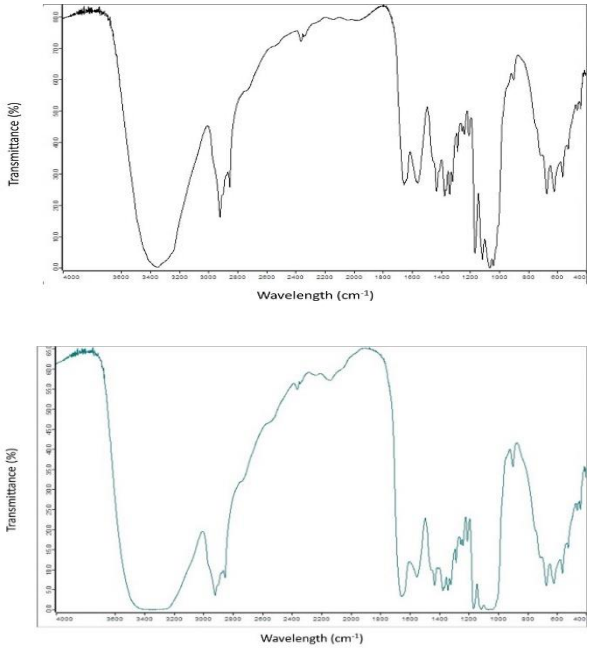


Figure 7. FTIR spectroscopy of **(a)** *K. intermedius*, **(b)** *K. maltaceti* and **(c)** *K. nataicola*.

4. Conclusion

The data obtained from this study indicate that propolis, at optimally determined concentrations for each *Komagataeibacter* species, contributed to BC production. However, higher concentrations of propolis inhibited BC production due to its antimicrobial properties. Additionally, bacterial consortia were found to enhance biopolymer production compared to the monocultures of the individual bacterial species and *K. intermedius* - *K. maltaceti* consortia is the most effective one. The increase in the volume of the production vessel was observed to enhance BC production due to the corresponding increase in nutritional content and surface area. Under static conditions, cellulose production was observed in membrane form, whereas under agitated conditions, irregularly shaped cellulose was formed. BC produced in HS broth containing propolis was found to have a high water-holding capacity (WHC) and moisture content ratio (MCR). The produced BC membranes were observed to have a three-dimensional nanofibrillar structure. As a result, it was concluded that the addition of propolis contributes to an increase in BC production efficiency.

Author's Contributions

Nermin Hande AVCIOGLU: Drafted and wrote the manuscript, performed the experiment and result analysis.

Ethics

There are no ethical issues after the publication of this manuscript.

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