Bacterial Nanocellulose from Symbiotic Culture of Bacteria and Yeast Kombucha Prepared with Lemongrass Tea and Sucrose: Optimization and Characterization

Pingkan Aditiawati, Intan Taufik, Julio Jonathan Gilbert Alexis, and Rudi Dungani *

Bacterial nanocellulose (BNC) can be produced using a variety of substrates as fermentation medium for use in various biomaterial applications. This study aimed to optimize the production of and characterize the BNC derived from lemongrass leaves (L-BNC) obtained by symbiotic culture of bacteria and yeast (SCOBY) kombucha. The lemongrass leaves (10, 15, and 20 g/L) and sugar (30, 50, and 70 g/L) were incubated for 14 d at 30 °C. The optimal treatment was used to ferment kombucha for 21 days at 30 °C, with initial SCOBY inoculum of 3% w/v and kombucha of 10% v/v for the resulting L-BNC. The L-BNC was characterized using scanning electron microscopy-energy dispersive X-ray (SEM-EDX) spectroscopy, Fourier-transform Infrared spectroscopy (FT-IR), and X-ray diffraction analysis (XRD). The optimal parameters of the lemongrass kombucha fermentation production process were lemongrass content 10 g/L and sugar content 30 g/L with the incubation period of two weeks for 56.8 g/L of SCOBY production. The SEM analysis of L-BNC revealed a three-dimensional fibrous extremely fine network of randomly arranged nanofibrils with diameter of 163 ± 34 nm and hydrogen bonds present in L-BNC fibril units. Meanwhile, XRD results showed a crystallinity of 67.2%.

DOI: 10.15376/biores.18.2.3178-3197

Keywords: Bacterial cellulose; Biomaterials; Lemongrass kombucha; Optimization; Nanocellulose

*Contact information: School of Life Sciences and Technology, Institut Teknologi Bandung, 40132, Indonesia; *Corresponding author: dunganir@gmail.com*

INTRODUCTION

Synthetic fibers are relatively expensive and require many harmful chemicals during production that impact humans and the environment. Conversely, bio-based materials, such as natural fibers, are more environmentally friendly and offer similar or better mechanical properties compared to traditional materials (de Azevedo *et al*. 2021). Fibers derived from bio-based sources, such as plant-based (ligno) cellulosics and protein-based fibers, are termed natural fibers. This includes natural cellulosic fibers, such as cotton, jute, sisal, coir, flax, hemp, abaca, ramie, *etc.*, and protein-based fibers such as wool and silk.

Cellulose is the most abundant natural polymer on earth; it is produced by diverse organisms, including plants, algae, oomycetes, and bacteria (Anderson *et al*. 2018; Park *et al*. 2019). One of the polymeric materials is bacterial cellulose (BC), which is produced by several microbial organisms (fungi and some bacterial species). The most studied species include *Komagataeibacter xylinus*, Gram-negative bacterium *Komagataeibacter* sp.,

Acetobacter sp., and *Gluconacetobacter* sp. (Moosavi-Nasab and Yousefi 2011; Abba *et al*. 2018). The BC from such organisms has shown superior physical properties convenient for many biomaterial applications such as biomedical (epithelial tissue graft), food (biodegradable food packaging), and materials engineering (composite technology) (Prudnikova and Shidlovsky 2017; Anton-Sales *et al*. 2019; Fernandes *et al*. 2019; Xu *et al*. 2021).

Because of their superior quality, such as high water-uptake and -holding capacity, high crystallinity, and high tensile strength with mechanical robustness (Lahiri *et al*. 2021), the enhancement of bacterial cellulose growth is currently being studied using different methods. *Gluconacetobacter kombuchae* is an acetic acid bacterium (AAB) known for effectively producing BC (Rahman *et al*. 2021). Different AAB strains showed their ability to synthesize BC from various carbon sources, including wastes, with varying production rates (Wang *et al*. 2018; Gorgieva and Trcek 2019; Merli *et al*. 2021). The achieved BC outputs differ in the significance level and range, which call for optimizing the biosynthetic technologies oriented to increase productivity.

In general, BC is produced in synthetic or non-synthetic media through oxidative fermentation media that contain a mixture of sugars with a particular concentration maintained at pH 3.0 and 28 °C through oxidative fermentation (Esa *et al*. 2014). During this process, the cellulose ribbons are formed from glucose chains of microfibrils inside the bacterial body that experienced aggregating. These ribbons generate a web-shaped network structure with plenty of empty spaces between the fibers (Shah *et al*. 2013; Esa *et al*. 2014). The production of BC is influenced by various factors, such as the culture medium, fermentation time, type of sugars, pH, temperature, and microbial diversity (Villarreal-Soto *et al*. 2019). Furthermore, the characterization of BC, such as crystallinity index, morphology, average crystallite size, and chemical compositions, as well as their mechanical properties, are determined by the fermentation process and the microbial strains (Gorgieva and Trcek 2019).

Several researchers have demonstrated the use of SCOBY kombucha as a starter and various substrates as the medium of cultivation. Aswini *et al*. (2020) reported that mango fruit is a good fermentation substrate because it has a higher BC yield and contains no impurities. Contrastingly, pH was effective for improving the yield of BC. The pH increased from 4.0 to 5.5 during the BC production phase in batch cultures by *Acetobacter xylinum.* These conditions improved cellulose production and the total fermentation time decreased (Hwang *et al*. 1999). Further study demonstrated that fructose, maltose, glycerol, xylose, starch, and nitrogen (in the form of casein hydrolysate and peptone), are the main components of the growth medium required for BC fermentation. As reported by Kim *et al*. (2006), using dual carbon sources (fructose and sucrose) produced around 8.79 g/L BC yield.

Kombucha is a traditional fermented drink made by fermenting sweetened tea using a symbiotic culture of bacteria and yeast (SCOBY) (Abel and Andreson 2020). During kombucha fermentation, a gelatinous, cellulose-based biofilm or a pellicle is formed at the air-liquid interface. The SCOBY consists of three major microbial groups, *i.e.*, yeasts, acetic acid bacteria (AAB), and lactic acid bacteria (LAB) (Tran *et al*. 2020; Kruk *et al*. 2021). The AAB in SCOBY synthetizes cellulose (BC) by polymerizing monosaccharides produced by yeast digestion of sugar (mainly sucrose). Meanwhile, LAB helps stabilize the overall microclimate conditions within the kombucha (Villarreal-Soto *et al*. 2018).

Lemongrass (*Cymbopogon citratus*) is an herbal plant containing several essential oils, phenolic compounds, and flavonoids (Ranjah *et al*. 2018). The chemical and

nutritional contents of lemongrass are similar to black tea and green tea, enabling its use as an alternative nutrient source for kombucha fermentation. Additionally, lemongrass can be cultivated in a much smaller plantation area than tea and can coexist with other plants, reducing production costs. Although the use of lemongrass in brewing kombucha is promising, several parameters could modify the BC yield such as substrate, temperature (Aswini *et al*. 2020), and fermentation time (Zhao *et al*. 2018).

Therefore, one important and challenging aspect of the fermentation process is identification of a new cost-effective culture medium that can facilitate the production of high yields within short periods of time, thereby improving BNC production and permitting application of BNC in the biotechnological, medical, pharmaceutical, and food industries.

Hence, the objective of this present study was to optimize the concentration of lemongrass, sugar content, temperature, and fermentation time that can produce the highest bacterial nanocellulose in lemongrass kombucha. The BNC fibers produced in the selected optimal condition were then characterized using scanning electron microscopy (SEM), Fourier-transform Infrared spectroscopy (FT-IR), and X-ray diffraction analysis (XRD).

EXPERIMENTAL

Pre-optimization of lemongrass and sugar concentration

Lemongrass leaves were ground using a food blender (BOLDe Super Food Processor Olympus, Shanghai, China) before they were boiled in distilled water for 15 min. The lemongrass tea filtrate was diluted to various concentrations (10, 15, and 20 g/L) before adding 50 g/L sugar (Jayabalan *et al*. 2014). A total of 200 mL lemongrass tea was poured into a dark plastic container. A starter culture that consisted of SCOBY (3% w/v) and kombucha (10% v/v) was inoculated to the brewed tea, and the container was covered with a piece of cheesecloth. Incubation was completed for 14 d at 30 °C. SCOBY was then filtered from kombucha to be weighed to determine the optimum lemongrass concentration.

After the optimal concentration of lemongrass tea was obtained, it was combined with three different sugar (99.9% of sucrose) concentrations: 30, 50, and 70 g/L (Jayabalan *et al*. 2014). The starter inoculum was sourced from the lemongrass kombucha used for lemongrass optimization. The SCOBY from this process was filtered, and weighed again as well as analyzed when the incubation period ended.

Preparation of optimized lemongrass kombucha

A total of 16 containers were used to incubate optimized lemongrass kombucha. Incubation was performed for 21 d at 30 °C. Two random containers were sampled once every three days for the chemical assays (once a week for single plateserial-dilution spotting (SP-SDS). Every consecutive sampling was performed on containers that had not yet been tested. This prevented the effect of physical disturbances on SCOBY colonizing the kombucha surface.

Analysis of SCOBY wet weight yield

The SCOBY was filtered away from the rest of the kombucha and weighed using an analytical weighing balance (OHAUS SPX421 Scout, OHAUS Corporation, Parsippany, NJ, USA). The weight measurement was performed in duplicate by taking two random containers from the incubator (Treviño-Garza *et al*. 2020).

Analysis of population dynamics

Each SCOBY was milled down with a food blender in a 1% peptone water (PW) solution (Harrison and Curtin 2021) and diluted to a factor of 10^{-5}. The kombucha was directly diluted to the same dilution factor. Each diluted solution was inoculated using the SP-SDS technique developed by Thomas *et al*. (2015) with three types of agar media: potato dextrose agar (PDA) + Penstrep-400® (final concentration of 125 U/mL) to isolate yeasts, MRSA (Methicillin-resistant *Staphylococcus aureus*) to isolate LAB, and GYCA (glucose yeast $CaCO_3$ agar) to isolate AAB. Both MRSA and GYCA were added with Nystatin® with a final concentration of 100 U/mL. Plates were incubated for 72 h at 30 °C, and colonies were purified before being identified using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany).

Analysis of total reducing sugar

An aliquot of the substrate kombucha (0.1 mL) was mixed with 9.9 mL of the distilled water. After 15 min of incubation at 95 °C, 0.5 mL of the dinitrosalicylic acid (DNS) reagent was added to the test tube and the mixture was incubated in a boiling water bath for 5 min. After cooling to room temperature, the absorbance of the supernatant at 540 nm was measured. The content of total sugar concentration was expressed as g of glucose equivalent (Başkan *et al*. 2016).

Analysis of total phenolic compound

The content of total phenolic compounds was determined by the Folin-Ciocalteu procedure. The reaction mixture was obtained by mixing 0.5 mL of kombucha, 4.5 mL of distillated water, and 0.5 mL Folin-Ciocalteu Assay. After 15 min of incubation at room temperature, the absorbance was measured at wavelength of 760 nm. The content of total phenolic compounds in investigated kombucha was expressed as g of gallic acid equivalents (GAE) per 100 g of the kombucha sample (%; w/w), *i.e.* %GAE (Velićanski *et al*. 2014).

Analysis of total organic acid

The kombucha samples were subjected to acid−base titration using a nominal 0.1 M sodium hydroxide (NaOH) solution standardized with 0.1 N acetic acid. The titration progress was monitored using a pH meter. Titrated acid was measured as acetic acid equivalent (AAE) (Velićanski *et al*. 2014).

Harvesting of bacterial nanocellulose

The whole fermentation process in the production of lemongrass bacterial cellulose used SCOBY inoculum 3% w/v and liquid inoculum (kombucha liquid) 10% v/v, without agitation, temperature 30 °C, initial pH of medium 5, during 21 d of fermentation. The SCOBY was soaked in pre-heated NaOH 0.5 M (90 °C) for 15 min before being soaked three times in boiling distilled water to remove cells, enzymes, and other metabolites and obtain pure lemongrass BC (L-BNC). Cleaned L-BNC was subsequently air-dried at room temperature for 72 h before determination of characterization.

Characteristics of L-BNC

SEM-energy dispersive X-ray (EDX)

The dried L-BNC sheet was mounted on a copper stub by double adhesive carbon conductive tape prior to coating with gold (SEM, SUPRA25 Carl Zeiss Company, Oberkochen, Germany) for 30 s. The characteristics of L-BNC were examined by a scanning electron microscope (Leo Supra, 50 VP, Carl Zeiss, SMT, Jena, Oberkochen, Germany) and operated at 15.0 kV at room temperature.

Fourier-transform infrared spectroscopy

The dried L-BNC pellicle sheet was cut to a size of 5 mm × 5 mm, before the transmission measurements were obtained using the FTIR spectrometer (Vertex 70, Bruker, Germany). Perkin Elmer spectrum 1000 was used to obtain the spectra of each sample, where 0.5 grams of sample was mixed with KBr (sample/KBr ratio 1/100). The spectra were recorded in the spectral region of 4000 to 400 cm^{-1} range with a resolution of 6 cm^{-1}.

X-ray diffraction

The X-ray diffraction patterns of L-BNC sheets were measured with a diffractometer (Philips PW1050 X-pert diffractometer, Westhorst, Germany) operating at 40 kV and 40 mA. The diffractograms were taken from 0 to 60° in a 2θ scale with a step size of 0.02°. Further, the crystallinity index (*CI*) was determined with the ratio of the area of all crystalline peaks (A_{cr}) to the total area (A_{total}) (Park *et al*. 2010), as shown in Eq. 1:

$$CI = A_{cr} / A_{total} \quad (1)$$

RESULTS AND DISCUSSION

Optimization Concentration of Lemongrass and Sugar

Lemongrass has been reported to produce a larger bacterial cellulose amount, when compared to banana peels (Sijabat *et al*. 2019). A concentration of 10 g/L lemongrass tea exhibited a maximum yield of SCOBY compared with another of two lemongrass concentrations, where the weight of SCOBY decreased with the increase of lemongrass concentrations, as shown in Fig. 1. Furthermore, the bacterial cellulose depended on the supply of a carbon source.

The experimental results showed that the concentration of sugar at 30g/L produced the highest yield of SCOBY and increasing sucrose concentration from (50 g/L to 70 g/L) produced a gradual decrease in the yield. Therefore, the optimal conditions of SCOBY yield generated were: 10 g/L lemongrass, 30 g/L of sugar, temperature (30 °C) and time (14 d). This yielded a SCOBY of 70.0 g/L. Hussin *et al*. (2015) reported a similar finding in the optimization study of tea fungus (Kombucha) beverage using natural sugars and organic acids. The authors mentioned that the optimum fermentation time of 14 d showed kombucha teas with color changes, decreased pH values, and total soluble solids.

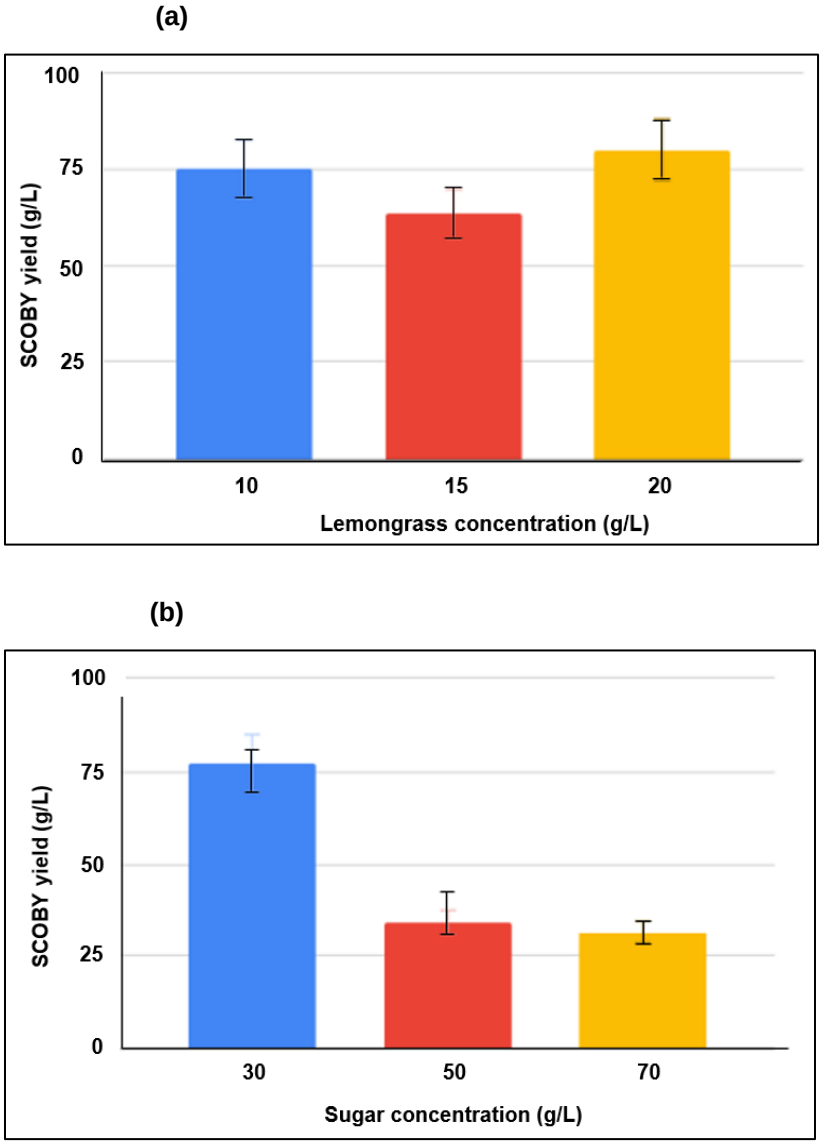


Fig. 1. SCOBY yield by incubated for 14 d at 30 °C. (a) at lemongrass concentrations; (b) at sugar concentrations

Analysis of SCOBY Wet Weight Yield

The SCOBY is defined as a microbial culture colonizing a complex BC matrix synthesized by acetic acid bacterium (AAB) in kombucha fermentation. The BC was synthesized using uridine diphosphate glucose (UDPG) as an intermediate. The UDPG can be obtained from various carbon sources such as glucose, fructose (through gluconeogenesis), ethanol, and glycerol. This flexibility enables AAB to balance and switch between multiple metabolic pathways to ensure a high yield of BC without interfering with its need for a carbon-derived energy source (Villarreal-Soto *et al*. 2019). Figure 2 shows the changes in wet weight of the SCOBY as the fermentation proceeded for 21 days. The SCOBY wet weight increased with fermentation time. The SCOBY wet yield increased progressively over from day 6 through 9, and there was a more gradual increase until the end of the incubation period (21 d). This shows an initial priority of

increasing cell numbers in the colonizing phase. Once an adequate cell number is achieved, AAB can now prioritize its carbon uptake to synthesize BC for further colonization by SCOBY. The BC production for SCOBY colonization on the kombucha surface is more convenient, as AAB is an obligate aerobe and access to oxygen is vital for BC synthesis.

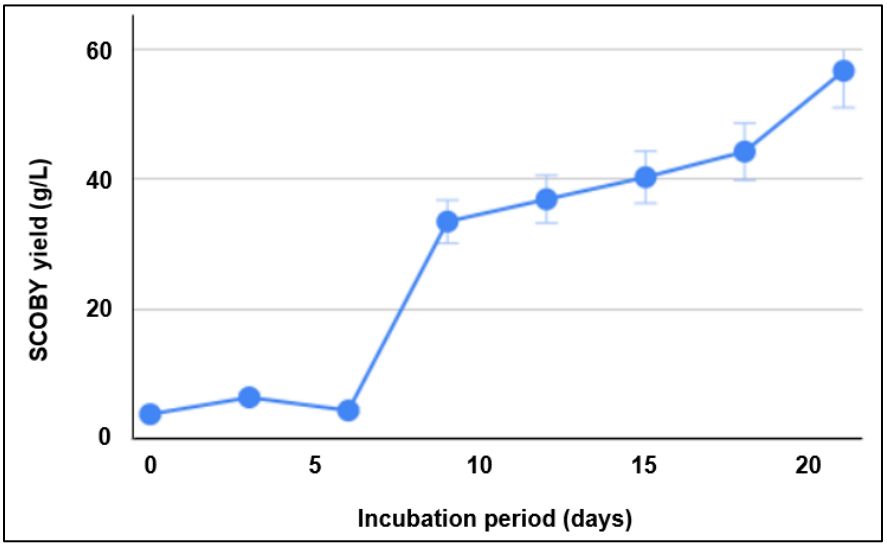


Fig. 2. SCOBY yield in optimized lemongrass kombucha incubated for 21 d at 30 °C

This result showed that pH increased slowly in co-culture compared to single culture cultivation, which could be the reason for lower BC yields. Furthermore, co-culture could promote synergistic growth but reduce BC yield (Devanthi *et al*. 2021). These results showed that fermentation may improve the biological activities of the media (tea or lemongrass) and that the production of bioactive compounds can vary depending on the fermentation conditions (Villarreal Soto *et al*. 2019).

Microbial Population Dynamics Analysis

Figure 3 shows that microbial population in SCOBY was higher than in the liquid phase (kombucha) after the lemongrass kombucha incubation process. Furthermore, both phases showed increased cell numbers in the first week, followed by a gradual decrease in over the rest of the incubation period. The authors' results also showed that within the bottom layer in SCOBY, the mean relative abundance of microbial appeared to vary according to location (inner, mid, or outer).

A possible explanation is that the microbial colonies prioritize cellular growth in the first week, then slow down the growth after the colony is established. Instead, it focuses on facilitating the synthesis of BC. The gradual decrease of cell numbers can be triggered by several factors, *e.g.*, a higher cell death compared to cell division, the thickening of SCOBY layers that made isolation more difficult, or accumulation of metabolic excrements such as excess organic acids. According to De Roos *et al*. (2018), solid phase (SCOBY) typically float to the surface of fermenting kombucha, where oxygen availability, temperature, and nutrient availability vary on either side to influence solid-phase formation and microbial community assembly. Oxygen limitation predominantly affects AAB-yeasts that are capable of facultative anaerobic respiration, while LABs are mostly aerotolerant anaerobic (Mamlouk and Gullo 2013).

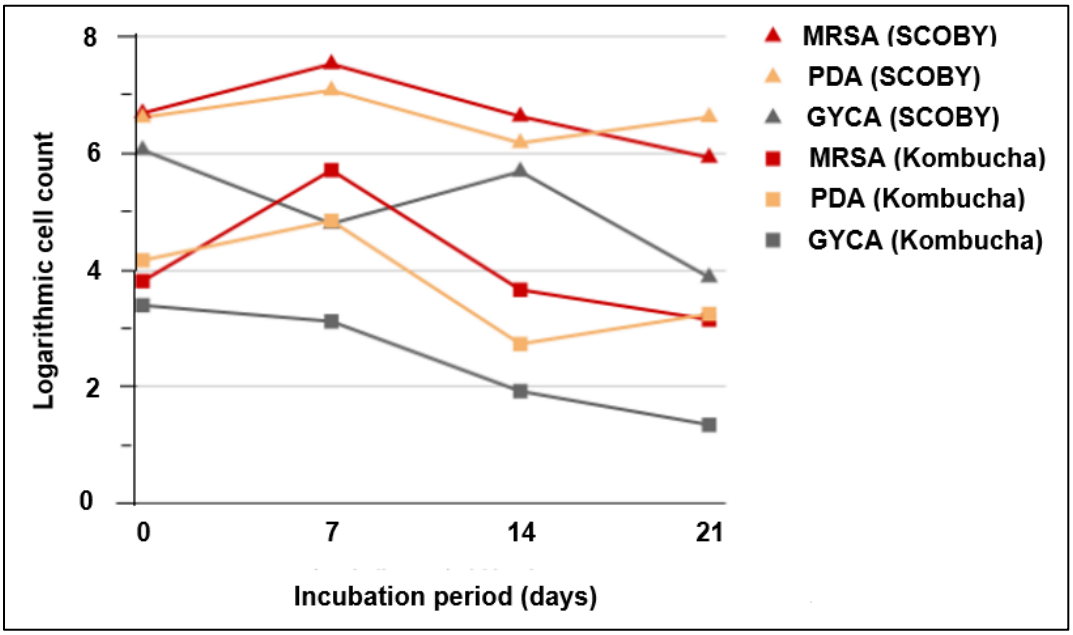


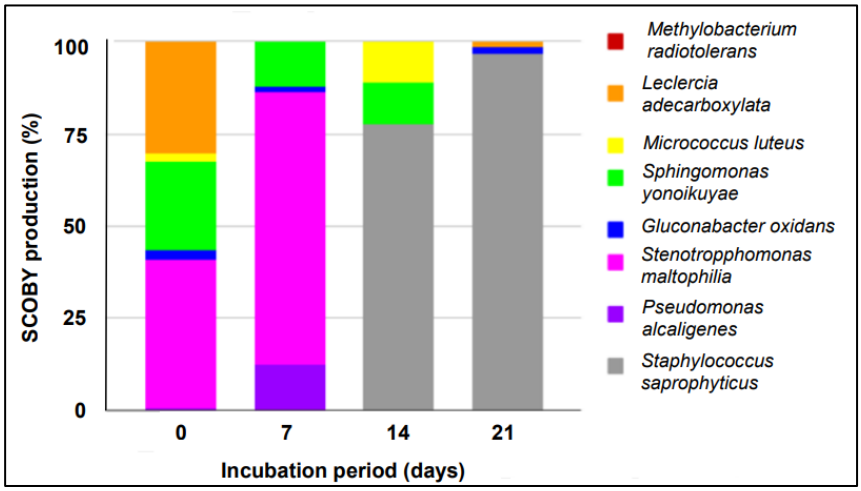
Fig. 3. Population dynamics within optimized lemongrass kombucha in a 21d incubation period at 30 °C

The GYCA exhibited the richest species counts, *i.e.*, eight in total, with *Gluconobacter oxydans* being the only AAB isolated (Fig. 4a). The AAB was not initially seen as a dominant species in kombucha fermentation despite being prominent. The AAB cells were embedded deeply inside the BC matrix, meaning the SCOBY must be ground thoroughly to isolate AAB and effectively give a more representative number. However, other isolates, such as *Pseudomonas alcaligenes* and *Sphingomonas yanoikuyae*, synthesize amorphous EPS layers, presenting further challenges in the isolation process. *Methylobacterium radiotolerans* can metabolize various carbon sources, enabling it to succeed in the later stages of the kombucha fermentation process when primary nutrient sources are scarce (Cordovana *et al*. 2019).

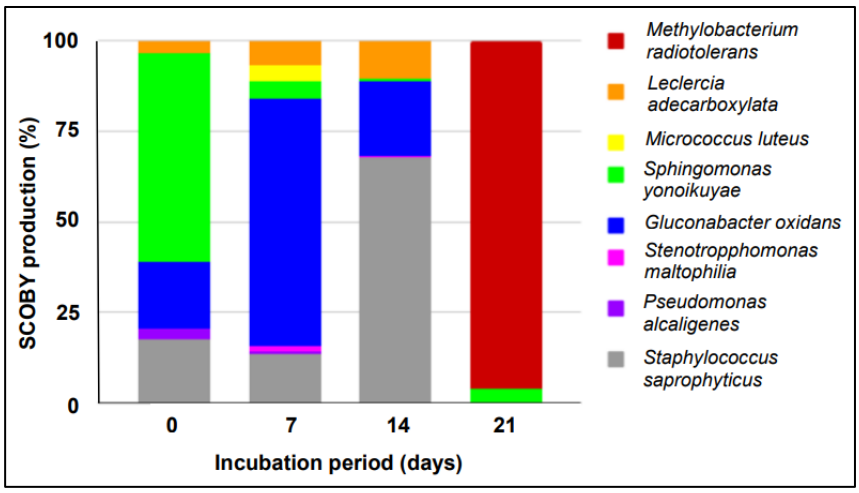
The results of the MRSA in Figs. 4c and 4d show that isolates of both liquid kombucha and solid SCOBY consisted of three unidentified species. They all formed dry, sticky, gel-like macroscopic colonies, causing difficulties in laser desorption/ionization, reducing the likelihood of gaining a high-confidence readout, eventually rendering the samples unidentifiable (Nie and Wang 2017). Based on the authors' observation, isolate L3 was the dominant species, followed by L2 and L1. Isolate L2 briefly became a dominant species on day 7 before eventually it fell behind isolate L3 and remained so until the end of the incubation period.

Two confirmed yeast species were isolated (Fig. 4e and 4f), *i.e.*, *Candida krusei* and *Zygosaccharomyces bisporus. C. krusei* is commonly found and used in cocoa fermentation. *Z. bisporus* has a high tolerance in extreme conditions (low pH, high acidity, low water activity, high sugar content, and high alcohol content) (Sharma and Sharma 2017). Although *C. krusei* has a high fermentative efficiency that enables it to dominate the initial fermentative processes, the microclimate in kombucha changes to support the total population of *Z. bisporus* in the later stages of fermentation.

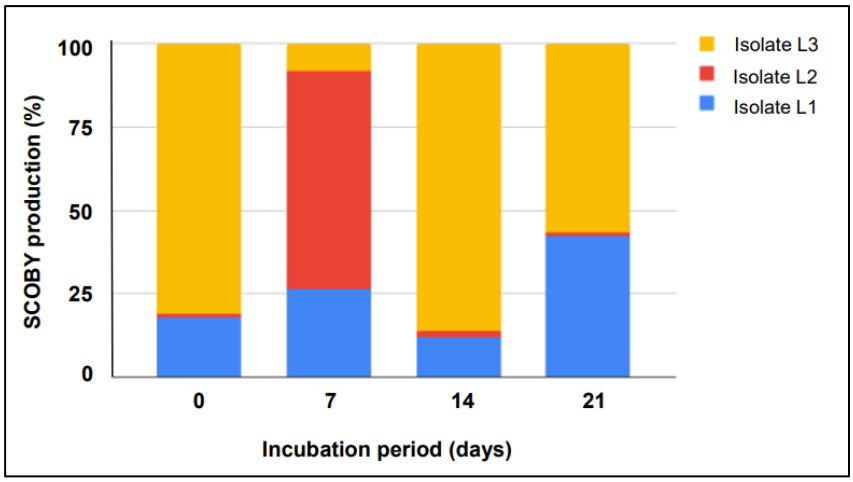
(a)


SCOBY production (%)
100
75
50
25
0
0
7
14
21
Incubation period (days)
Methylobacterium radiotolerans
Leclercia adecarboxylata
Micrococcus luteus
Sphingomonas yonoikuyae
Gluconabacter oxidans
Stenotropphomonas maltophilia
Pseudomonas alcaligenes
Staphylococcus saprophyticus

(b)


SCOBY production (%)
100
75
50
25
0
0
7
14
21
Incubation period (days)
Methylobacterium radiotolerans
Leclercia adecarboxylata
Micrococcus luteus
Sphingomonas yonoikuyae
Gluconabacter oxidans
Stenotropphomonas maltophilia
Pseudomonas alcaligenes
Staphylococcus saprophyticus

(c)


SCOBY production (%)
100
75
50
25
0
0
7
14
21
Incubation period (days)
Isolate L3
Isolate L2
Isolate L1

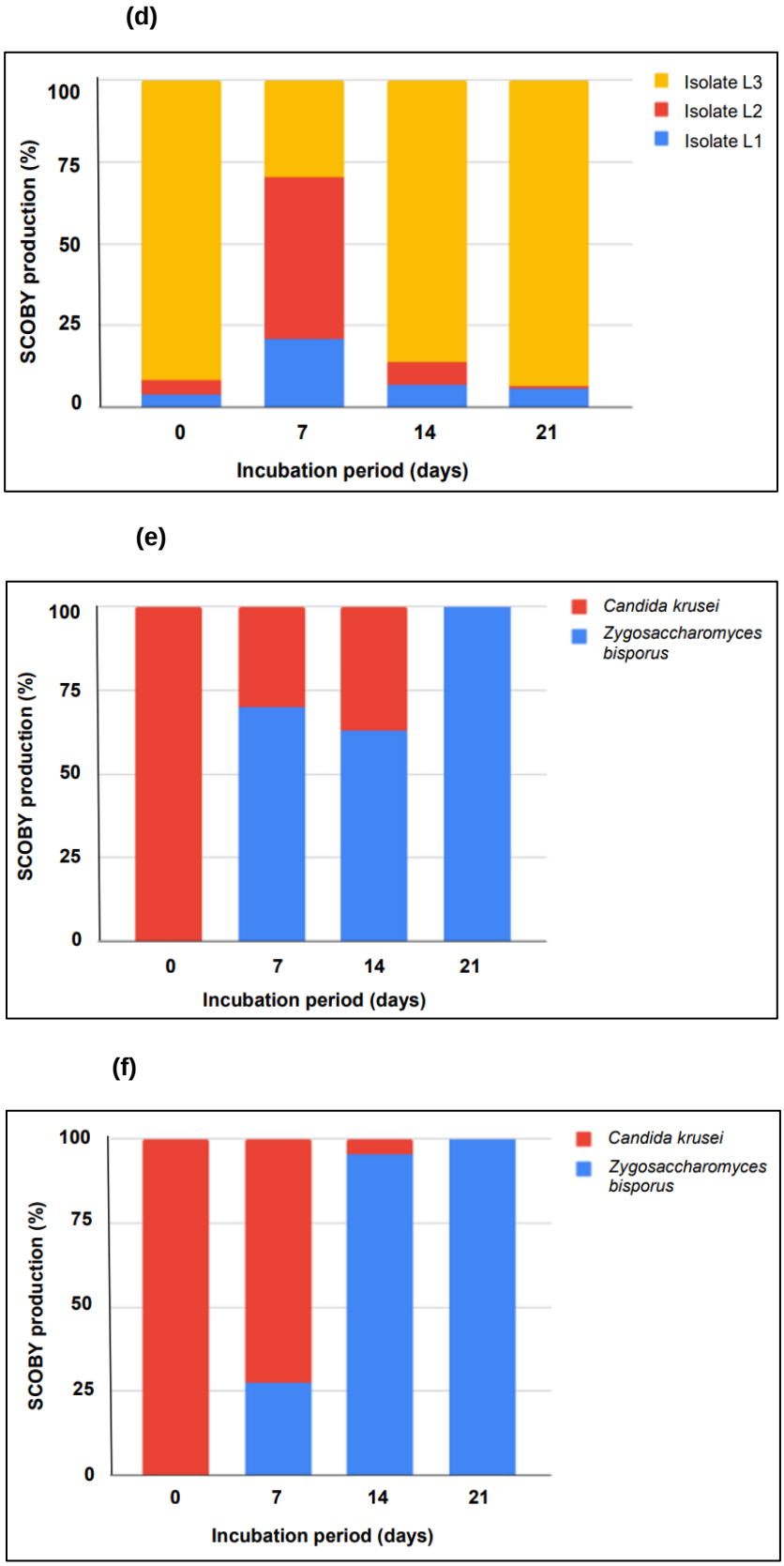
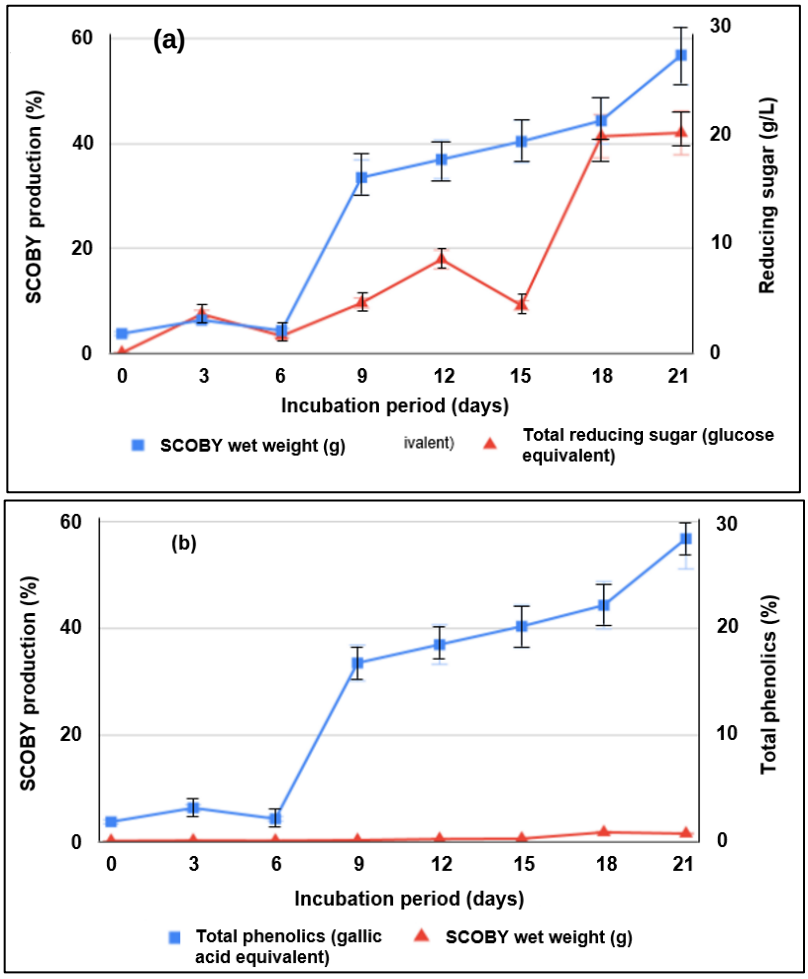


Fig. 4. Microbial population dynamics: (a) GYCA isolates in kombucha matrix; (b) GYCA isolates in SCOBY matrix; (c) MRSA isolate in kombucha matrix; (d) MRSA isolate in SCOBY matrix; (e) PDA isolate in kombucha matrix; (f) PDA isolate in SCOBY matrix

Total Reducing Sugar, Total Phenolics, and Total Organic Acids

The saccharification of sucrose by yeasts in optimized lemongrass kombucha resulted in two reducing sugars in the form of monosaccharides (glucose and fructose). Both monosaccharides were used primarily as a source of energy for the microbes and building blocks for AAB to synthesize BC. Glucose is the default go-to carbon source. The content of reducing sugar in kombucha fluctuates throughout the incubation period because of the monosaccharide uptake rate and the saccharification rate (Fig. 5a). Overall, reducing sugar content increased from 0.018 ± 0.064 g/L to 20.159 ± 8.034 g/L. This result showed that the conversion rate from sugar to BNC and acid was high within 21 d. A higher reducing sugar consumption can break down sucrose into reducing sugars, increasing their availability to microbes (Timmermans *et al*. 2022). The amount of reducing sugars left in produced kombucha implies that the fermentation process may still be able to continue beyond 21 d, as suggested because SCOBY's wet weight still increases by the end of the graph (Fig. 5a).



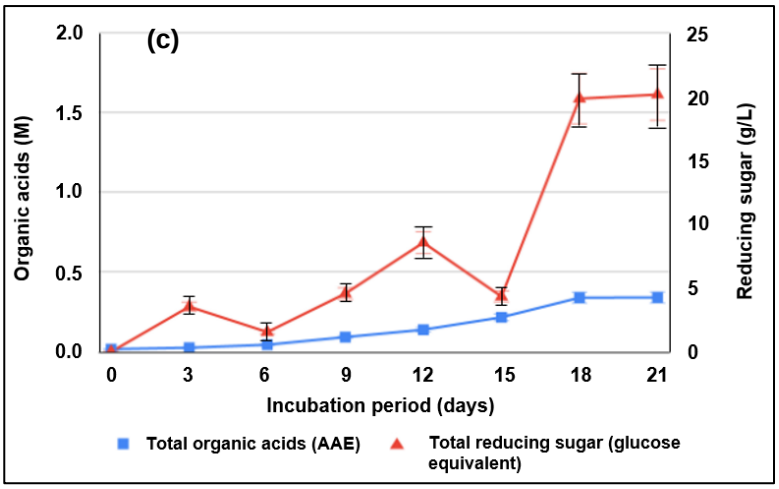


Fig. 5. Production of total chemical components at 30 °C: (a) total reducing sugar; (b) total incubation period of 21 d at total phenolics; (c) total organic acids compared to production of reducing sugar

Phenolic compounds in kombucha are primarily sourced from the degradation of complex phenolics, such as lignin, from the lemongrass into simple phenolics (Fig. 5b). The presence of antioxidant activities can also accelerate this degradative process. Phenolic metabolism in lemongrass kombucha was minimal until day 15 of the incubation period, followed by a slight increase from day 15 through 18 (Fig. 5c), which means that phenolic metabolism was not a primary metabolism in kombucha. Phenolic metabolism metabolizes alternative energy sources and/or as a stress response by the microbes (Mamlouk and Gullo 2013). Total organic acids in lemongrass kombucha increased from 0.018 ± 0.001 M to 0.342 ± 0.163 M (Fig. 5c). This suggests that a lot of monosaccharides were left unfermented. Likewise, the unfermented monosaccharides resulted from an uneven rate of saccharification and fermentation, particularly in the late stage of the fermentation process (Arez *et al.* 2014). Oxygen limitations slowed down the fermentation by AAB and LAB. In general, these bacterial cellulose systems have limited acid tolerance. At the same time, *Z. bisporus* continued to break down the remaining sugar because of their high survivability in such an adverse environment.

Characteristics of L-BNC

Figure 6 shows the scanning electron micrographs of L-BNC produced using kombucha fermentation yields by SCOBY. The surface structure of L-BC in the form of a porous film was characterized by a three-dimensional fibrous extremely fine network of randomly arranged nanofibrils and hydrogen bonds present in L-BNC fibril units (Fig. 6a). Furthermore, the L-BNC SEM image shows a complex, multi-layered microfibril network with diameter of 163 ± 34 nm (Fig. 6b). According to Costa *et al*. (2017), BC film produced by *Gluconacetobacter hansenii* showed results similar to nanofibrils forming a homogenous, compact, and three-dimensional structure. Literature identified that soaking SCOBY in NaOH removed cells, enzymes, and other metabolites applied for destructuring the cellulose matrix into high-valued cellulose nano- and microfibrils (Dima *et al*. 2017). The arrangement and alignment of the microfibril affect the crystallinity index (Daicho *et al*. 2018). Contrastingly, the pore size and volume percentage of hollow space within the matrix affect the structural strength, flexibility, and cellular accessibility (Hakim *et al*. 2017; Razzaz and Rodrigue 2018; Zhou *et al*. 2019).

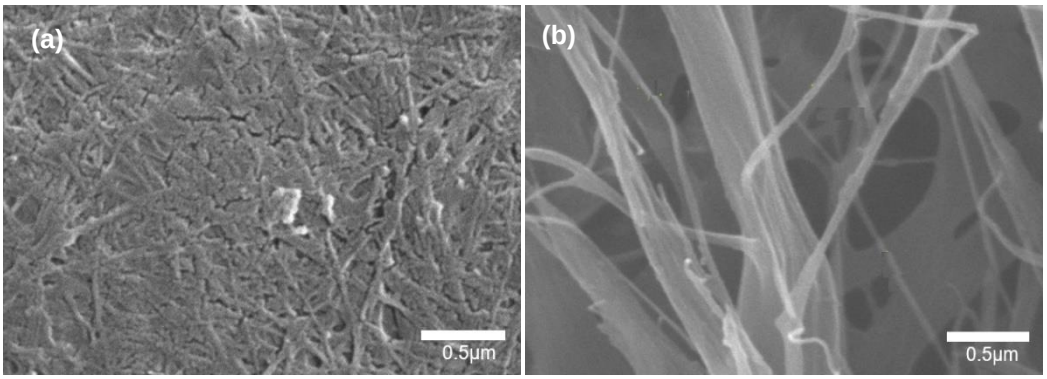


Fig. 6. Fracture surface of L-BNC film: (a) 3D ultrafine network of L-BNC; (b) Analysis of diameter of the L-BNC

Elemental analysis % (w/w) composition data of L-BNC from SEM-EDX is shown in Table 1. The X-ray diffraction profile of L-BNC is shown in Fig. 7. The EDX spectra of L-BNC show four distinctive elements. The L-BNC contained a relatively high percentage of carbon and oxygen, and also a small amount of sodium and calcium. The high percentage of carbon and oxygen found was also shown in other studies (Gutierrez *et al*. 2013; Pogorelova *et al*. 2020), because they are the two primary constituents of cellulose. The EDX analysis is not able to detect hydrogen atoms because it only has a single electron and electron 'skin'. It was likely that sodium and calcium were detected because of residual metabolic material and formations of the mineral silica, respectively.

L-BNC has many different groups and bonds that can be characterized using FT-IR. The FTIR spectra for L-BNC produced by SCOBY are shown in Fig. 8. According to the literature, as a polysaccharide, the presence of β 1→4 glycosidic bonds (at 1161 cm^{-1}) is key in determining that the sample is cellulose (Güzel and Akpınar 2018).

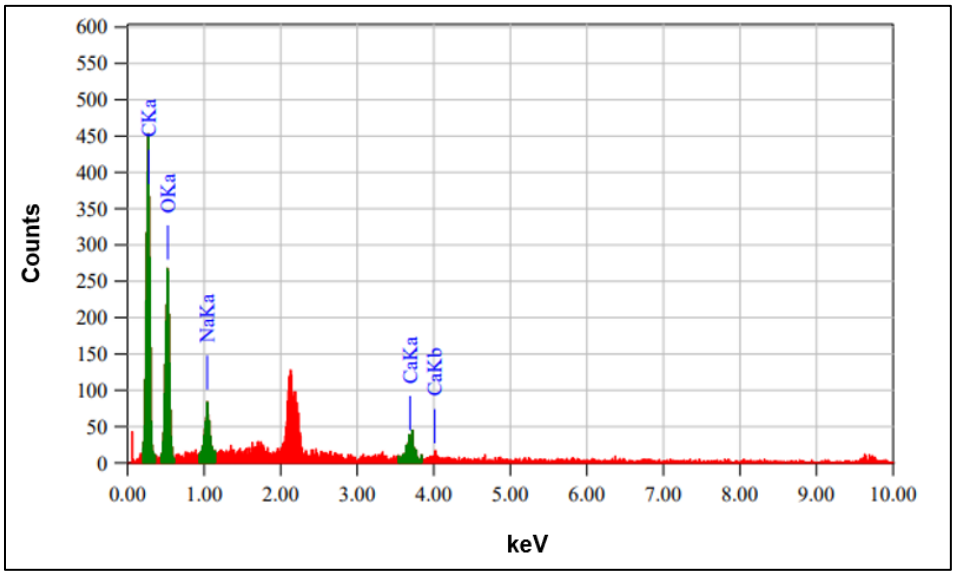


Fig. 7. EDX spectra of extracted L-BNC showing four detected elements

Table 1. Elemental Composition of Extracted BC

Elements	(keV)	Mass %	Error %	Atom %
C	0.277	52.47	0.07	60.72
O	0.525	42.36	0.28	36.80
Na	1.041	2.66	0.12	1.61
Ca	3.690	2.51	0.24	0.87
Total		100.00		100.00

Two steep V-shaped peaks between 2200 to 3800 cm^{-1} frequency revealed the alkane bond with a hydroxyl group and hydrogen bond, which confirms the absence of the benzene ring group found in lignin. The presence of a benzene ring forms a sloping U-shaped peak. The peak at 1587.42 cm^{-1} and 1658 cm^{-1} determines the presence of the amine/amide group. These are not typically found in cellulose samples and are considered a contaminant, meaning that the extraction needs to be completed more thoroughly (Fuller *et al*. 2018). Overall, the obtained FT-IR spectra of L-BNC produced by SCOBY is consistent with other reports, where the bacterial cellulose produced by *Komagataeibacter hansenii*, *Gluconacetobacter hansenii*, and *Acetobacter xylinum*. The structure of bacterial cellulose was the same because the bands of absorption appeared at the same wavenumbers (Takai *et al*. 1975; Park *et al*. 2003; Güzel and Akpınar 2018).

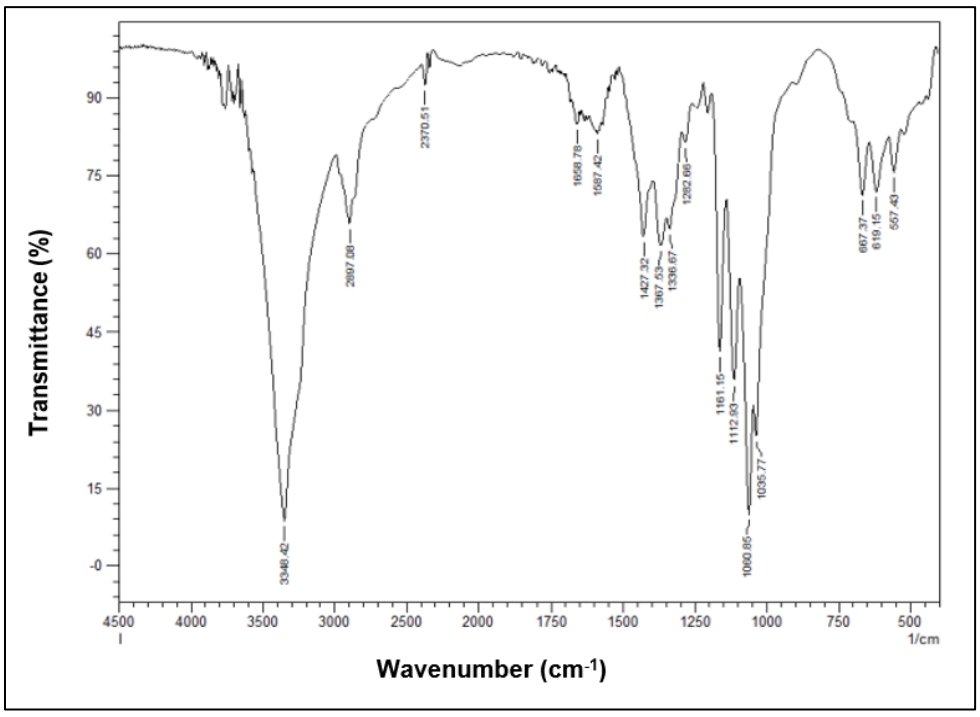


Fig. 8. FT-IR spectra of L-BNC sample

Typical results of the XRD spectra of the L-BNC samples are provided in Fig. 9. As shown, in the XRD diffraction pattern of L-BNC samples the three strongest peaks were at the diffraction angles 2θ of 14.6°, 18.6°, and 23.7º, while the low intensity peaks were assigned to other elements present in L-BNC. The CI of L-BNC was calculated from XRD intensity data using peak deconvolution method. The XRD spectroscopic results in

Fig. 9 were deconvoluted to obtain the CI. The sample did not show any crystalline transformation in the L-BNC structure; however, it had different crystallinity levels.

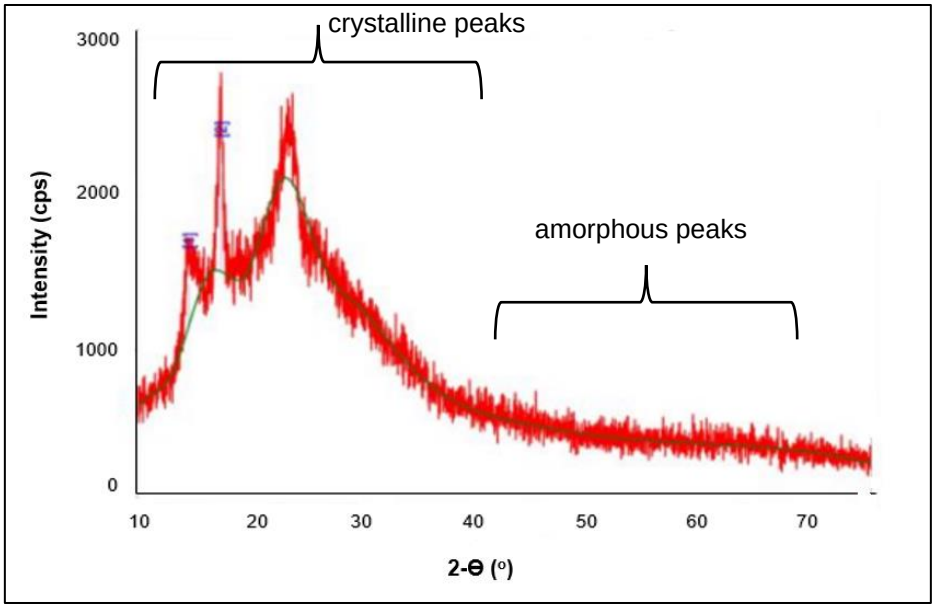


Fig. 9. XRD spectroscopy of L-BNC sample

The L-BNC had a CI of 67.2%, which is comparable with literature data for BC biosynthesized by *Gluconacetobacter xylinus*, ranging from 65% to 75% (Ruan *et al*. 2016). There were many factors, such as cultivation method, carbon sources, pH, temperature, fermentation time, and drying methods, affecting the crystallinity of BC. In the current study, the reduced carbon source from lemongrass extract might strongly influence the enhanced properties of BC by providing the fructose and glucose required for bacterial growth (Gomes *et al*. 2013; Güzel and Akpınar 2018).

CONCLUSIONS

1. Bacterial cellulose prepared with a culture of lemongrass and sucrose (L-BNC) was successfully isolated from lemongrass leaves through symbiotic culture of bacteria and yeast (SCOBY) kombucha.
2. The conditions for optimizing the kombucha fermentation production process were at temperature of 30 °C for incubation period of two weeks, with a composition of 10 g/L lemongrass, and 30 g/L white sugar.
3. The L-BNC showed the presence of C, O, Na, and Ca. The main surface functional groups present in L-BNC were ring forms similar to aromatic and amine/amide groups.
4. The scanning electron microscope (SEM) analysis revealed a three-dimensional fibrous extremely fine network of randomly arranged nanofibrils with diameter of 163 ± 34 nm. The crystallinity was 67.2%.

ACKNOWLEDGMENTS

The researchers would like to thank the Ministry of Education, Culture, Research, and Technology (KEMENDIKBUDRISTEK), Indonesia for providing research Grant No. 2/E1/KP. PTNBH/2021 that made this work possible. The authors also thank the Regional Health Laboratory of West Java (LABKESDA, Jawa Barat) for assisting and troubleshooting the MALDI-TOF MS procedure.

REFERENCES CITED

Abba, M., Abdullahi, M., Nor, M. H. M., Chong, C. S., and Ibrahim, Z. (2018). "Isolation and characterisation of locally isolated *Gluconacetobacter xylinus* BCZM sp. with nanocellulose producing potentials," *IET Nanobiotechnol*. 12(1), 52-56. DOI: 10.1049/iet-nbt.2017.0024

Abel, M., and Andreson, H. (2020). "Effect of simultaneous inoculation of commercial yeast starter cultures on kombucha fermentation," *Agron. Res*. 18(S3), 1603-1615. DOI: 10.15159/AR.20.140

Anderson, L. A., Islam, M. A., and Prather, K. L. J. (2018). "Synthetic biology strategies for improving microbial synthesis of "green" biopolymers," *J. Biol. Chem*. 293(14), 5053-5061. DOI: 10.1074/jbc.TM117.000368

Anton-Sales, I., Beekmann, U., Laromaine, A., Roig, A., and Kralisch, D. (2019). "Opportunities of bacterial cellulose to treat epithelial tissues," *Curr. Drug Targets*. 20(8), 808-822. DOI: 10.2174/1389450120666181129092144

Arez, B. F., Alves, L., and Paixão, S. M. (2014). "Production and characterization of a novel yeast extracellular invertase activity towards improved dibenzothiophene biodesulfurization," *Appl. Biochem. Biotechnol.* 174(6), 2048-2057. DOI: 10.1007/s12010-014-1182-x

Aswini, K., Gopal, N. O., and Uthandi, S. (2020). "Optimized culture conditions for bacterial cellulose production by *Acetobacter senegalensis* MA1," *BMC Biotechnol*. 20, article 46. DOI: 10.1186/s12896-020-00639-6

Başkan, K. S., Tütem, E., Akyüz, E., Özen, S., and Apak, S. (2016). "Spectrophotometric total reducing sugars assay based on cupric reduction," *Talanta* 147, 162-168. DOI: 10.1016/j.talanta.2015.09.049

Cordovana, M., Deni, A., Kostrzewa, M., Abdalla, M., and Ambretti, S. (2019). "First report of *Methylobacterium radiotolerans* bacteraemia identified by MALDI-TOF mass spectrometry," *New Microbes New Infect.* 30, article ID 100546. DOI: 10.1016/j.nmni.2019.100546

Costa, A. F. S., Almeida, F. C. G., Vinhas, G. M., and Sarubbo, L. A. (2017). "Production of bacterial cellulose by *Gluconacetobacter hansenii* using corn steep liquor as nutrient sources," *Front. Microbiol*. 8, article 2027. DOI: 10.3389/fmicb.2017.02027

Daicho, K., Saito, T., Fujisawa, S., and Isogai, A. (2018). "The crystallinity of nanocellulose: Dispersion-induced disordering of the grain boundary in biologically structured cellulose," *ACS Appl. Nano. Mater*. 1(10), 5774-5785. DOI: 10.1021/acsanm.8b01438

de Azevedo, A. R. G., Cruz, A. S. A., Marvila, M. T., de Oliveira, L. B., Monteiro, S. N., Vieira, C. M. F., Fediuk, R., Timokhin, R., Vatin, N., and Daironas, M. (2021).

"Natural fibers as an alternative to synthetic fibers in reinforcement of geopolymer matrices: A comparative review," *Polymers* 13(15), article ID 2493. DOI: 10.3390/polym13152493

De Roos, J., Vandamme, P., and De Vuyst, L. (2018). "Wort substrate consumption and metabolite production during lambic beer fermentation and maturation explain the successive growth of specific bacterial and yeast species," *Front. Microbiol.* 9, article ID 2763. DOI: 10.3389/fmicb.2018.02763

Devanthi, P. V. P., Kho, K., Nurdiansyah, R., Briot, A., Taherzadeh, M. J., and Aslanzadeh, S. (2021). "Do kombucha symbiotic cultures of bacteria and yeast affect bacterial cellulose yield in molasses?," *J. Fungi* 7(9), article 705. DOI: 10.3390/jof7090705

Dima, S. O., Panaitescu, D. M., Orban, C., Ghiurea, M., Doncea, S. M., Fierascu, R. C., Nistor, C. L., Alexandrescu, E., Nicolae, C. A., Trică, B., *et al.* (2017). "Bacterial nanocellulose from side-streams of kombucha beverages production: Preparation and physical-chemical properties," *Polymers* 9(8), article 374. DOI: 10.3390/polym9080374

Esa, F., Tasirin, S. M., and Rahman, N. A. (2014). "Overview of bacterial cellulose production and application," *Agric. Agric. Sci. Proc.* 2, 113-119. DOI: 10.1016/j.aaspro.2014.11.017

Fernandes, M., Gama, M., Dourado, F., and Souto, A. T. (2019). "Development of novel bacterial cellulose composites for the textile and shoe industry," *Microb. Biotechnol.* 12(4), 650-661. DOI: 10.1111/1751-7915.13387

Fuller, M. E., Andaya, C., and McClay, K. (2018). "Evaluation of ATR-FTIR for analysis of bacterial cellulose impurities," *J. Microbiol. Methods.* 144, 145-151. DOI: 10.1016/j.mimet.2017.10.017

Gomes, F. P., Silva, N. H. C. S., Trovatti, E., Serafim, L. S., Duarte, M. F., Silvestre, A. J. D., Neto, C. P., and Freire, C. S. R. (2013). "Production of bacterial cellulose by *Gluconacetobacter sacchari* using dry olive mill residue," *Biomass Bioenerg.* 55, 205-211. DOI: 10.1016/j.biombioe.2013.02.004

Gorgieva, S., and Tr˘cek, J. (2019). "Bacterial cellulose: Production, modification and perspectives in biomedical applications," *Nanomater.* 9, article 1352. DOI: 10.3390/nano9101352

Gutierrez, J., Fernandes, S. C. M., Mondragon, I., and Tercjak, A. (2013). "Multifunctional hybrid nanopapers based on bacterial cellulose and sol–gel synthesized titanium/vanadium oxide nanoparticles," *Cellulose* 20(3), 1301-1311. DOI: 10.1007/s10570-013- 9898-2

Güzel, M., and Akpınar, Ö. (2018). "Production and characterization of bacterial cellulose from citrus peels," *Waste Biomass Valor.* 10, 2165-2175. DOI: 10.1007/s12649-018-0241-x

Hakim, I., Donaldson, S., Meyendorf, N., and Browning, C. (2017). "Porosity effects on interlaminar fracture behavior in carbon fiber-reinforced polymer composites," *Mater. Sci. Appl*. 8, 170-187. DOI: 10.4236/msa.2017.82011

Harrison, K., and Curtin, C. (2021). "Microbial composition of SCOBY starter cultures used by commercial kombucha brewers in North America," *Microorganisms* 9(5), article ID 1060. DOI: 10.3390/ microorganisms9051060

Hussin, A. S. M., Wan Sapawi, C. W. N. S. C., Osman, F. A., Muhamad, R., and Sukor, R. (2015). "Effects of sugar sources and incubation time on the properties of tea

fungus (Kombucha) beverage," *J. Probiotics Health* 3(3), 66-69. DOI: 10.4172/23298901.C1.021

Hwang, J.-W., Yang, Y.-K., Hwang, J.-K., Pyun, Y.-R., and Kim, Y.-S. (1999). "Effects of pH and dissolved oxygen on cellulose production by *Acetobacter xylinum* BRC5 in agitated culture," *J. Biosci. Bioeng.* 88(2), 183-188. DOI: 10.1016/s13891723(99)80199-6

Jayabalan, R., Malbaša, R. V., Lončar, E. S., Vitas, J. S., and Sathishkumar, M. (2014). "A review on kombucha tea microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus," *Compr. Rev. Food Sci. Food Saf.* 13(4), 538-550. DOI: 10.1111/1541-4337.12073

Kim, S.-Y., Kim, J.-N., Wee, Y.-J., Park, D.-H., and Ryu, H. W. (2006). "Production of bacterial cellulose by *Gluconacetobacter sp*. RKY5 isolated from persimmon vinegar," *Appl. Biochem. Biotechnol.* 131, 705-715. DOI: 10.1385/ABAB:131:1:705

Kruk, M., Trzaskowska, M., Scibisz, I., and Pokorski, P. (2021). "Application of the "SCOBY" and kombucha tea for the production of fermented milk drinks," *Microorganisms* 9(1), article 123. DOI: 10.3390/microorganisms9010123

Lahiri, D., Nag, M., Dutta, B., Dey, A., Sarkar, T., Pati, S., Edinur, H.A., Abdul Kari, Z., Mohd Noor, N.H., and Ray, R.R. (2021). "Bacterial cellulose: Production, characterization, and application as antimicrobial agent," *Int. J. Mol. Sci*. 22, 12984. DOI: 10.3390/ijms222312984

Mamlouk, D., and Gullo, M. (2013). "Acetic acid bacteria: Physiology and carbon sources oxidation," *Indian J. Med. Microbiol*. 53(4), 377-384. DOI: 10.1007/s12088013-0414-z

Moosavi-Nasab, M., and Yousefi, M. (2011). "Biotechnological production of cellulose by *Gluconacetobacter xylinus* from agricultural waste," *Iran J. Biotechnol*. 9(2), article ID 94101.

Merli, G., Becci, A., Amato, A., and Beolchini, F. (2021). "Acetic acid bioproduction: The technological innovation change," *Sci. Total Environ.* 798, article ID 149292. DOI: 10.1016/j.scitotenv.2021.149292

Park, J. K., Park, Y. H., and Jung, J. Y. (2003). "Production of bacterial cellulose by *Gluconacetobacter hansenii* PJK isolated from rotten apple," *Biotechnol. Bioprocess Eng*. 8, 83-88. DOI: 10.1007/BF02940261

Park, M.-A., Lee, S.-G., Choi, K.-H., and Cho, B.-U. (2019). "Effects of surface coating of cellulose II-based MFC on paper properties," *Pulp and Paper Technology* 51(1), 28-36. DOI: 10.7584/JKTAPPI.2019.02.51.1.28

Park, S., Baker, J. O., Himmel, M. E., Parilla, A. P., and Johnson, K. D. (2010). "Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance," *Biotechnol. Biofuel.* 3, article 10. DOI: 10.1186/1754-68343-10

Pogorelova, N., Rogachev, E., Digel, I., Chernigova, S., and Nardin, D. (2020). "Bacterial cellulose nanocomposites: Morphology and mechanical properties," *Materials* 13(12), 2849-2864. DOI: 10.3390/ma13122849

Prudnikova, S. V., and Shidlovsky, I. P. (2017). "The new strain of acetic acid bacteria *Komagataeibacter xylinus* B-12068 – Producer of bacterial cellulose for biomedical applications," *J. Sib. Fed. Univ. Biol*. 10(2), 246-254. DOI: 10.17516/1997-13890017

Rahman, S. S. A., Vaishnavi, T., Sai Vidyasri, G., Sathya, K., Priyanka, P., Venkatachalam, P., and Karuppiah, S. (2021). "Production of bacterial cellulose using

Gluconacetobacter kombuchae immobilized on *Luffa aegyptiaca* support," *Sci. Rep.* 11, article 2912. DOI: 10.1038/s41598-021-82596-4

Ranjah, M. A., Waseem, M., Taj, F., and Ashraf, I. (2018). "Lemongrass (*Cymbopogon citratus*): A valuable ingredient for functional foods," *Int. J. Food Allied Sci*. 4(2), 11-19. DOI: 10.21620/ijfaas.2018211-19

Razzaz, Z., and Rodrigue, D. (2018). "Hollow fiber porous nanocomposite membranes produced via continuous extrusion: Morphology and gas transport properties," *Mater*. 11(11), article 2311. DOI: 10.3390/ma11112311

Ruan, C., Zhu, Y., Zhou, X., Abidi, N., Hu, Y., and Catchmark, J. M. (2016). "Effect of cellulose crystallinity on bacterial cellulose assembly," *Cellulose* 23, 3417-1342. DOI: 10.1007/s10570-016-1065-0

Shah, N., Ul-Islam, M., Khattak, W. A., and Park, J. K. (2013). "Overview of bacterial cellulose composites: A multipurpose advanced material," *Carbohydr. Polym.* 98(2), 1585-1598. DOI: 10.1016/j.carbpol.2013.08.018

Sharma, A., and Sharma, S. C. (2017). "Physiological basis for the tolerance of yeast *Zygosaccharomyces bisporus* to salt stress," *HAYATI J. Biosci.* 24(4), 176-181. DOI: 10.1016/j.hjb.2017.11.001

Sijabat, E. K., Nuruddin, A., Aditiawati, P., and Purwasasmita, B. S. (2019). "Synthesis and characterization of bacterial nanocellulose from banana peel for water filtration membrane application," *J. Phys.: Conference Series*, 1230(1). DOI: 10.1088/1742-6596/1230/1/012085

Takai, M., Tsuta, Y., Hayashi, J., and Watanabe, S. (1975). "Biosynthesis of cellulose by *Acetobacter xylinum*. III. X-ray studies of preferential orientation of the crystallites in a bacterial cellulose membrane," *Polym. J.* 7, 157-164. DOI: 10.1295/polymj.7.157

Timmermans, E., Bautil, A., Brijs, K., Scheirlinck, I., Van der Meulen, R., and Courtin, C. M. (2022). "Sugar levels determine fermentation dynamics during yeast pastry making and its impact on dough and product characteristics," *Foods* 11(10), article 1388. DOI: 10.3390/foods11101388

Tran, T., Grandvalet, C., Verdier, F., Martin, A., Alexandre, H., and Tourdot-Maréchal, R. (2020). "Microbial dynamics between yeasts and acetic acid bacteria in kombucha: Impacts on the chemical composition of the beverage," *Foods* 9(7), article 963. DOI: 10.3390/foods9070963

Treviño-Garza, M. Z., Guerrero-Medina, A. S., González-Sánchez, R. A., García-Gómez, C., Guzmán-Velasco, A., Báez-González, J. G., and Márquez-Reyes, J. M. (2020). "Production of microbial cellulose films from green tea (*Camellia sinensis*) kombucha with various carbon sources," *Coatings* 10(11), article 1132. DOI: 10.3390/coatings10111132

Velićanski, A. S., Cvetković, D. D., Markov, S. L., Tumbas Šaponjac, V. T. T., and Vulić, J. J. (2014). "Antioxidant and antibacterial activity of the beverage obtained by fermentation of sweetened lemon balm (*Melissa officinalis* L.) tea with symbiotic consortium of bacteria and yeasts," *Food Technol. Biotechnol*. 52(4), 420-429. DOI: 10.17113/ftb.52.04.14.3611

Villarreal-Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J. P., and Taillandier, P. (2018). "Understanding kombucha tea fermentation: A review," *J. Food Sci*. 83(3), 580-588. DOI: 10.1111/1750-3841.14068

Villarreal Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J. P., Renard, T., Rollan, S., and Taillandier, P. (2019). "Impact of fermentation conditions on the production of bioactive compounds with anticancer, anti-inflammatory and antioxidant properties in kombucha tea extracts," *Process Biochem*. 83, 44-54. DOI: 10.1016/j.procbio.2019.05.004

Xu, Y., Liu, X., Jiang, Q., Yu, D., Xu, Y., Wang, B., and Xia, W. (2021). "Development and properties of bacterial cellulose, curcumin, and chitosan composite biodegradable films for active packaging materials," *Carbohydr. Polym*. 15(260), 223-236. DOI: 10.1016/j.carbpol.2021.117778

Wang, S. S., Han, Y. H., Chen, J. L., Zhang, D. C., Shi, X. X., Ye, Y. X., Chen, D. L., and Li, M. (2018). "Insights into bacterial cellulose biosynthesis from different carbon sources and the associated biochemical transformation pathways in *Komagataeibacter* sp. W1," *Polymers* 10, article 963. DOI: 10.3390/polym10090963

Zhao, H., Xia, J., Wang, J., Yan, X., Wang, C., Lei, T., Xian, M., and Zhang, H. (2018). "Production of bacterial cellulose using polysaccharide fermentation wastewater as inexpensive nutrient sources," *Biotechnol. Biotechnol. Equip*. 32(2), 350-356. DOI: 10.1080/13102818.2017.1418673

Zhou, J., Kang, T., and Wang, F. (2019). "Pore structure and strength of waste fiber recycled concrete," *J. Eng. Fibers Fabrics* 14, 1-10. DOI: 10.1177/1558925019874701

Article submitted: December 17, 2022; Peer review completed: January 14, 2023; Revised version received and accepted: January 24, 2023; Published: March 10, 2023.
DOI: 10.15376/biores.18.2.3178-3197