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**Abstract**

This work explores the production of kombucha-derived bacterial cellulose (KBC) from sour whey via the fermentation method using Komagatacibacter xylinus. The biosynthesis process was optimized by design of experiments and the results displayed highest KBC yield at 1000 ml/L sour whey waste, 87.39 g/L cane sugar, 6 g/L black tea, and 78.91 ml/L bacteria volume under 21 days culture period at 30°C. Optimum fermentation batch efficiency was achieved in large scale with cultured medium depths of 0.5 cm and low-residual bacteria suspension volume of 72.31 ± 8.74 ml. The obtained KBC membranes were analyzed by SEM, FTIR, XRD, and TGA. The obtained results show no significant differences for all prepared KBC samples when compared to pristine bacterial cellulose from standard Hestrin and Schramm (HS) medium. In addition, the optimized KBC was investigated as a suitable biofiller in the preparation of biocomposite materials. The prepared biocomposites as leather alternative were further characterized and their mechanical tensile strength and elongation at break determined in the range of 135.61 ± 9.15 to 154.89 ± 9.09 N/mm$^2$ and 31.06 ± 0.32 to 92.33 ± 6.91%, respectively. This model obtained depicts high-yield production of KBC and its potential in the preparation of biocomposites.

**KEYWORDS**: Microscopy, morphology, phase behavior

## 1 INTRODUCTION

Cleaner production and sustainable development have gradually become the necessary standards of manufacturing industries[1,2] that have constantly been spreading worldwide and have crept into the consuming and trading habits of individuals.[3-5] For example, leather-related industries such as fashion design, textiles, footwear, bags, or interior covering products in recent years have witnessed drastic reforms in terms of their production methods to suit the extremely trendy-progress toward sustainable materials.[6,7] This is because of conventional manufacturing stages such as pre-processing, bleaching/ tanning, degreasing, deodorizing, decoloring, shaping, and product processing causes countless risks to the envi-ronmental.[7,8] As such, the development of eco-friendly related leather materials by the incorporation of biobased polymers as reinforcing additives have shown great research interest as

potential ingredients that may correctly and fully respond to such urgent requirements.[6,9-11] Among all, bacterial cellulose (BC) has demonstrated to be a viable reinforcing ingredient source for the preparation of alternative leather-like materials. This is attributed to its excellent biocompatibility, environmental friendliness, no toxicity, thermal stability, and high-mechanical properties (Young modulus; 15-20 GPa, tensile strength; 200-300 MPa).[12-15] In addition, BC is abundant and widely produced from the fermentation of countless by-products of dairy foods and agroforestry processings.[16-22] This makes BC cost-effective and sustainable with readily available raw material sources for production as compared to cellulose obtained from plants that causes hazards to the environment via deforestation.

Several studies have been explored for the production of BC using nutrient solutions of kombucha beverage through short-term fermentation of sugar and tea both at home kitchen conditions as well as at research laboratories using kits or equipments.[10,17,23-25] These produced BC types are currently known as kombucha-derived bacterial cellulose (KBC) with very close physicochemical and mechanical properties to BC obtained from standard Hestrin and Schramm (HS) medium[26] as well as other nutrient mediums.[10,19,21,25] Nevertheless, both BC and KBC show low-yields that somehow hampers the commercial application level of these green-material sources with no sufficient degree to explore at high capacity.[20,27,28] In the effort to address this obstacle, different isolation techniques and using high-yield fermentation bacterial strains or alternative nutrient sources (carbon and nitrogen) have been explored as solutions to significant increase cellulose yield during biosynthesis.[16,19,29-35] However, within the context of a circular economy and sustainable development trend, the liquid organic waste treatment ability of the KBC fermentation method is also necessary to pay attention to take advantage of its dual benefits (improving cellulose biosynthesis yield, while handled the emission sources).

Dairy whey liquid waste is one of the most polluting by-products of the food industry. This generated waste is made of 50-55% total milk nutrients that may serve as a potential extra-nutrient source for strong growth stimulation of KBC during biosynthesis.[20,36-38] Typically, the large-scale application potential of a trial or fermentation batch is first evaluated following yield and cost-effectiveness. As such, the present study focuses on enhancing KBC yield by adding dairy sour whey waste as an additional nutrient source/ingredient into the fermentation medium followed by optimization of the production process. All the factors investigated strongly adhered to the requirement of simplicity maximization toward achieving the best results. According to literature, *Komagataeibacter xylinus (formerly called Acetobacter xylinum or Gluconacetobacter xylinus*) has been known as the most efficient cellulose-producing bacterial [strain16,39-41] that has been used as the only fermentation strain to replace Kombucha symbiotic microbiota. While, cane sugar is a potential carbon source that has replaced glucose thereby limiting gluconic acid formation that lowers the cultured medium pH, leading to inhibition of cellulose biosynthesis capacity using *Komagatacibacter xylinus*.[13,25,42-44] For this reason, omitting the pH adjustment step greatly simplifies the process toward application in industrial production.

Design of experiment (DoE) is a multipurpose statistical tool for optimal formulation and has been widely applied in the industrial sectors of pharmaceutics, biomedicine, cosmetics, textile, food, and agriculture.[16,31,39,45-49] Herein, DoE was applied to optimize the biosynthesis of KBC from dairy industrial wastes. As such, sour whey and black tea were used in the present study as alternatives to expensive components such as yeast extract and peptone to provide nitrogen and some enhancers (albumins, globulins, vitamins, amino acids, and organic acids) for bacterial cell-respiration.[17,20,24,25,36,37,42,50] Normally, KBC membranes are formed as gel-like three-dimensional materials at the top surface of the culture medium under static conditions,[17,23,25,51] and as such different factors including amount of sour whey, cane sugar content, black tea, bacteria volume, and number of culture days are investigated to

determine the optimum conditions. In addition, a determinant experiment based on efficiency of cultured medium depth for fermentation in large scale was evaluated to minimize the amount of residual suspension since it presents itself as the only source of emissions during KBC production. Thereafter, biocomposite leather-like materials were fabricated by homogenously blending the newly obtained optimized KBC membranes with treated sisal fiber, polyurethane (PU) and polylactic acid (PLA) to confirm the effectiveness of the approach of turning bio-waste sources into material wealth. The obtained KBC membranes and prepared biocomposite materials were then characterized for their structural, morphological, thermal resistance, surface wettability, and mechanical strength.

## 2 EXPERIMENTAL SECTION

### *2.1 Materials*

Sour whey was collected from Kromilk A.S (Kromeriz, Czech Republic). Cane sugar and black tea were purchased from a grocery store in Zlin, Czech Republic. Yeast extract, peptone, hexane, dichorodimethylsilane (DCDMS) and 4-(dimethylamino)pyridine (DMAP) were supplied by Sigma-Aldrich (Darmstadt, Germany). Polylactic acid (PLA-4043D) and PU, used as a matrix additive were supplied by NatureWorks LLC (Ingeo). D-glucose was supplied by Amersco LLC (MA). Sodium hydroxide (NaOH), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$) and citric acid ($C_6H_8O_7$) were purchased from Penta s.r.o. (Zlin, Czech Republic). Standard grade sisal fiber was supplied by Wigglesworth, Co, Ltd, (London, UK).

### *2.2 Activation of bacterial strain*

Komagatacibacter xylinus CCM 3611 was supplied by the Microbiology Laboratory of the Centre of Polymer Systems, University Institute, Tomas Bata University in Zlin (Czech Republic). Prior to use, the bacterial strain was activated on standard HS medium as described by Schramm and Hestrin[26] to obtain a suspension of approximately 1.8 x $10^{10}$ CFU/ml.

### *2.3 Experimental design*

Culture mediums components are measured, directly mixed, and contained in Duran bottles (250 ml capacity). Each trial formulation was performed with 100 ml sterile medium (sterilized at 121° C, 1 atm, 15 min) in triplicates under static cultured condition at 30°C. Response surface methodology (RSM) based on D-optimal (custom) design consisting of 28 trials with five survey factors coded by five independent variables was used to design the experiments, analyze data and develop regression models. Therefore, four factors including sour whey [A], cane sugar [B], black tea [C], and bacteria volume [D] were randomly conducted at continuous high and low concentrations. While, the other factor designated as culture period [E] was investigated at three discrete concentration levels of high, medium, and low to reach the integer values of 14, 18, and 21 days. By slight modification, these concentration levels have been confirmed as the fit parameter ranges to achieve high BC yield in recent similar studies.[16,31,33,34,39,40,52]

**TABLE 1** Corresponding volume depths of the fermentation mediums in large containers

| Samples | Cultured medium depth (cm) | Cultured medium volume (ml) |
|---|---|---|
| KBC-0.5 cm | 0.5 | 180 |
| KBC-1 cm | 1 | 360 |
| KBC-2 cm | 2 | 720 |
| KBC-3 cm | 3 | 1080 |
| HS-0.5 cm | 0.5 | 180 |
| HS-1 cm | 1 | 360 |
| HS-2 cm | 2 | 720 |
| HS-3 cm | 3 | 1080 |

*Abbreviations: HS, Hestrin and Schramm; KBC, kombucha-derived bacterial cellulose.*

The standard HS medium was prepared as the control. Variable [D] was then transferred to the respectively cultured medium in a sterile cabinet. The dry weight of KBC membranes was fixed as the dependent variable.

The results were subjected to analysis of variance (ANOVA) with a significance level of $\alpha = 0.05$. The correlation between the dependent variable and the independent variables were integrated via nonlinear regression analyses. Finally, three verification experiments were performed to confirm the optimum conditions.

### *2.4 Determination of cultured medium depth in large containers*

Typically, large containers of same dimensions (17 x 25 cm ~ S = 425 $cm^2$) were cleaned with 70% ethanol and the prepared sterile fermentation medium from the obtained optimized formulation was poured into these containers following various volumes to produce different corresponding cultured medium depths as shown in **Table 1**.

Standard HS medium was prepared as the control. The fermentation process was then maintained for 21 days under static conditions at 30° C, after which the generated cellulose membranes were collected, treated, and the dry weight determined.

**TABLE 2** Constituent components in the preparation of leather-like materials

| Samples | KBC alkali-treated (% wt/wt) | KBC-treated (% wt/wt) | Fiber treated (% wt/wt) | Polyurethane (PU) (% wt/wt) | Polylactic acid (PLA) (% wt/wt) |
|---|---|---|---|---|---|
| S1 | 5 | – | – | 65 | 30 |
| S2 | – | 5 | – | 65 | 30 |
| S3 | – | 10 | – | 60 | 30 |
| S4 | – | 15 | – | 60 | 25 |
| S5 | – | 5 | 20 | 50 | 25 |
| S0 (Control) | – | – | – | 65 | 35 |

*Abbreviation: KBC, kombucha-derived bacterial cellulose.*

*2.5 Fabrication of the biocomposites (leather-like materials)*

Initially, harvested KBC membranes and sisal fibers were treated by immersion in 0.5% NaOH wt/vol for 1 h, followed by washing severally with $dH_2O$ and blended vigorously at 30000 rpm for 1 min using the NutriBullet blender (N17-0908 machine). The obtained dried treated KBC and sisal fiber was further modified with dichlorodimethylsilane (DCDMS) for 1 h using 4-(dimethylamino)pyridine (DMAP) as the catalyst, washed with n-hexan and ethanol:dH2O (1:1). The final samples were then oven-dried at 40° C to constant weight and grinder for 1 min using a micro ball mill (Lab Wizz 320, Laarmann Group, Netherlands) at room temperature and a frequency rate of 25 Hz. The biocomposite materials were subsequently fabricated via blending of modified KBC and sisal fibers in various ratios with PU and PLA, as shown in **Table 2**.

**TABLE 3** The experimental designe for the optimization of KBC culture

| | Experimental factors | | | | | Medium pH | | Response KBC dry weight (g/L) | |
|---|---|---|---|---|---|---|---|---|---|
| Run | Factor A sour whey (ml/L) | Factor B cane sugar (g/L) | Factor C black tea (g/L) | Factor D bacteria volume (ml/L) | Factor E culture period (days) | Pre-culture | After culture | Actual | Predicted |
| 1 | 1000.00 | 100.00 | 6.00 | 100.0 | 21 | 5.9 | 4.6 | 20.06 | 20.09 |
| 2 | 652.50 | 50.00 | 3.00 | 73.0 | 14 | 5.7 | 4.8 | 16.01 | 15.91 |
| 3 | 1000.00 | 50.00 | 3.00 | 100.0 | 21 | 5.9 | 4.9 | 18.05 | 17.99 |
| 4 | 500.00 | 100.00 | 3.00 | 10.0 | 14 | 5.7 | 4.4 | 12.84 | 12.77 |
| 5 | 1000.00 | 100.00 | 3.00 | 100.0 | 14 | 5.6 | 4.7 | 16.15 | 16.10 |
| 6 | 1000.00 | 50.00 | 3.00 | 100.0 | 21 | 5.7 | 4.9 | 17.90 | 17.99 |
| 7 | 1000.00 | 50.00 | 6.00 | 100.0 | 14 | 6.0 | 4.9 | 14.68 | 14.73 |
| 8 | 500.00 | 100.00 | 6.00 | 100.0 | 14 | 5.7 | 4.5 | 14.56 | 14.48 |
| 9 | 1000.00 | 100.00 | 3.00 | 48.7 | 18 | 5.9 | 4.6 | 17.83 | 17.73 |
| 10 | 750.00 | 50.00 | 4.58 | 10.0 | 18 | 5.8 | 4.9 | 15.01 | 15.03 |
| 11 | 500.00 | 50.00 | 6.00 | 10.0 | 14 | 5.7 | 4.9 | 12.04 | 12.06 |
| 12 | 787.50 | 78.50 | 3.00 | 10.0 | 21 | 5.7 | 4.7 | 17.00 | 17.03 |
| 13 | 1000.00 | 100.00 | 4.26 | 10.0 | 21 | 5.7 | 4.6 | 17.25 | 17.21 |
| 14 | 500.00 | 50.00 | 6.00 | 100.0 | 21 | 6.0 | 4.7 | 17.23 | 17.25 |
| 15 | 1000.00 | 100.00 | 6.00 | 10.0 | 14 | 5.7 | 4.6 | 14.22 | 14.21 |
| 16 | 1000.00 | 75.00 | 4.58 | 55.0 | 14 | 5.7 | 4.7 | 16.09 | 16.04 |
| 17 | 500.00 | 100.00 | 6.00 | 10.0 | 21 | 5.8 | 4.4 | 16.63 | 16.64 |
| 18 | 500.00 | 100.00 | 3.00 | 100.0 | 21 | 6.0 | 4.5 | 15.26 | 15.17 |
| 19 | 1000.00 | 100.00 | 3.00 | 48.7 | 18 | 5.7 | 4.6 | 17.63 | 17.73 |
| 20 | 710.00 | 90.00 | 4.73 | 61.3 | 21 | 5.9 | 4.6 | 18.62 | 18.65 |
| 21 | 1000.00 | 50.00 | 6.00 | 10.0 | 21 | 5.7 | 4.9 | 16.06 | 16.00 |
| 22 | 500.00 | 68.75 | 3.00 | 100.0 | 18 | 5.8 | 4.6 | 16.99 | 17.07 |
| 23 | 1000.00 | 50.00 | 3.00 | 10.0 | 14 | 5.7 | 4.9 | 13.05 | 13.10 |
| 24 | 804.97 | 61.75 | 4.71 | 100.0 | 18 | 5.8 | 4.9 | 18.23 | 18.13 |
| 25 | 1000.00 | 100.00 | 4.26 | 10.0 | 21 | 5.7 | 4.7 | 17.17 | 17.21 |
| 26 | 1000.00 | 100.00 | 6.00 | 10.0 | 14 | 5.7 | 4.7 | 14.18 | 14.21 |
| 27 | 500.00 | 50.00 | 3.00 | 10.0 | 21 | 5.9 | 4.7 | 15.15 | 15.14 |
| 28 | 570.00 | 100.00 | 3.42 | 87.4 | 14 | 5.9 | 4.5 | 14.01 | 14.61 |
| HS | 20 g D-glucose, 5 g yeast extract, 5 g peptone, 8.6 g $Na_2HPO_4.12H_2O$ and 1.15 g citric acid | | | | | 5.8 | 3.8 | 4.96 | – |

*Abbreviations: HS, Hestrin and Schramm; KBC, kombucha-derived bacterial cellulose.*

The components were mixed at room temperature, to form a thick mixture, and compressed with a force of 5-20 kN using a 2 mm thick stainless at 150° C. Before each compression, the samples were placed between Teflon sheets. After pressing, samples were air-dried at room temperature for 24 h and used for further analysis.

*2.6 Treatment and determination of cellulose membranes (dry weight)*

Harvested cellulose membranes were washed with distilled water in triplicate, blotted with tissue paper, oven dried at 40° C to constant weight and weighed for the determination of dry weights, using the mathematical formulation Equation (1):

$$Dry\,weight = \frac{\text{Weight of membrane after oven drying (g)}}{\text{Volume of medium (L)}}. \quad (1)$$

On the other hand, harvested cellulose membranes were washed with distilled water, treated with 0.5% wt/vol NaOH at 80°C for 1 h, rewashed with distilled water, blotted with tissue paper, freeze-dried at —110° C for 24 h, and stored in a desiccator for further analyses.

*2.7 pH value of nutrient mediums*

The pH values of the nutrient mediums and suspension were determined using an electronic handheld pH meter (Lovibond pH meter-445R).

*2.8 Characterization techniques*

*2.8.1 Scanning electron microscopy*

The microstructure of the samples was observed using the scanning electron microscope (SEM) (FEI, Brno, Czech Republic), operating at an accelerating voltage of 2 kV and imaging distance of 6-7 mm using a program ImageJ (NIH) to measure the diameter of cellulose fibers.

*2.8.2 Fourier transformed infrared spectroscopy*

The chemical groups of the samples were analyzed on Fourier transformed infrared (FT-IR) spectrophotometer (Nicolet iS5, Thermo Scientific) attached with an attenuated total reflection mode (iD5-Ge-ATR). FT-IR spectrum was acquired at a 4 $cm^{-1}$ resolution using 64 scans in the range of 400-4000 $cm^{-1}$ at room temperature.

### *2.8.3 Thermogravimetric analysis*

The thermal stability attributes of the samples were recorded using a Q500 (TGA) thermogravimetric analyzer (TA Instruments). The samples were weighed in the range of 6.5-7.0 mg, heated from 25 to 600°C under nitrogen atmosphere at a heating rate of 10°C/min and a flow rate of 40-60 ml/min.

### *2.8.4 X-ray diffraction analysis*

X-ray diffraction (XRD) analysis was conducted using a Mini Flex 600 X-ray diffractometer (Rigaku, Japan) to measure the crystalline nature of the samples. The scans were performed in the range of 0-90°, at a speed of 5°/ min using a foil filtered CuKβ radiation (λ = 0.179 nm) at 40 kV voltage and a current of 15 mA.

**TABLE 4** Analysis of variance (ANOVA) for the applied response surface quadratic model

| Source | Sum of squares | Df | Mean square | F-value | *p*-value | |
|---|---|---|---|---|---|---|
| Model | 98.11 | 20 | 4.91 | 24.55 | <0.0001 | Significant |
| Residual | 0.139 | 7 | 0.020 | | | |
| Lack of fit | 0.103 | 3 | 0.034 | 3.79 | 0.1152 | Not significant |
| Pure error | 0.036 | 4 | 0.009 | | | |
| Cor total | 98.25 | 27 | | | | |
| $R^2$ | | | | 0.998 | | |
| Adjusted $R^2$ | | | | 0.994 | | |
| Predicted $R^2$ | | | | 0.894 | | |
| Adeq precision | | | | 65.57 | | |

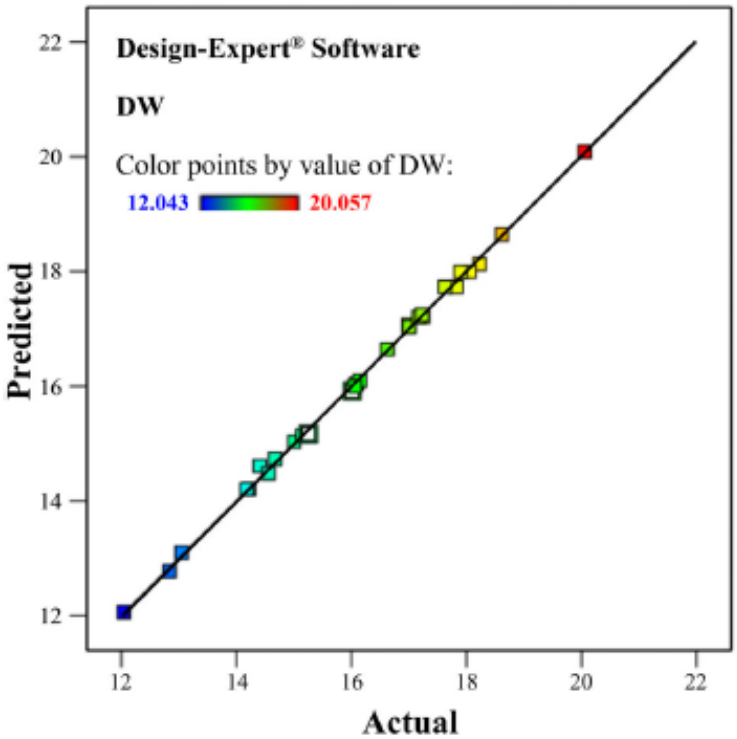


**FIGURE 1** Predicted versus actual for cellulose membrane dry weights [Color figure can be viewed at wileyonlinelibrary.com]

The divergence slit was maintained at 0.1° throughout the experiment. The crystallinity index percentage (CI%) was calculated using Equation (2).[33]

$$CI\% = \frac{I_{002} - I_{am}}{I_{002}} X\,100, \qquad (2)$$

where, *I002* is the maximum intensity of the 002-crystal plane reflection of cellulose *I* and $I_{am}$ is the maximum intensity of X-ray scattering broadband related to the amorphous part of the samples.

*2.8.5 Mechanical analysis*

Instron 5567 apparatus (Instron) was used to conduct the tensile tests as per ISO 37 standards[53] with a static load of 5 kg. Five specimens of each sample were tested at a crosshead speed of 10 mm/min at room temperature.

*2.8.6 Surface wettability measurements*

The sessile drop technique was used to measure the contact angle for the wettability determination of the prepared biocomposites using Advex Instrument machine (Brno, Czech Republic) with a charged-coupled device (CCD) camera. The measurements were conducted at 25° C and 50% relative humidity. Distilled water was used as the checking liquid with 10 µl for each testing. The photographic images were taken after the deposition liquid was dropped on the surface of the sample. The measured result was an average of five replicates.

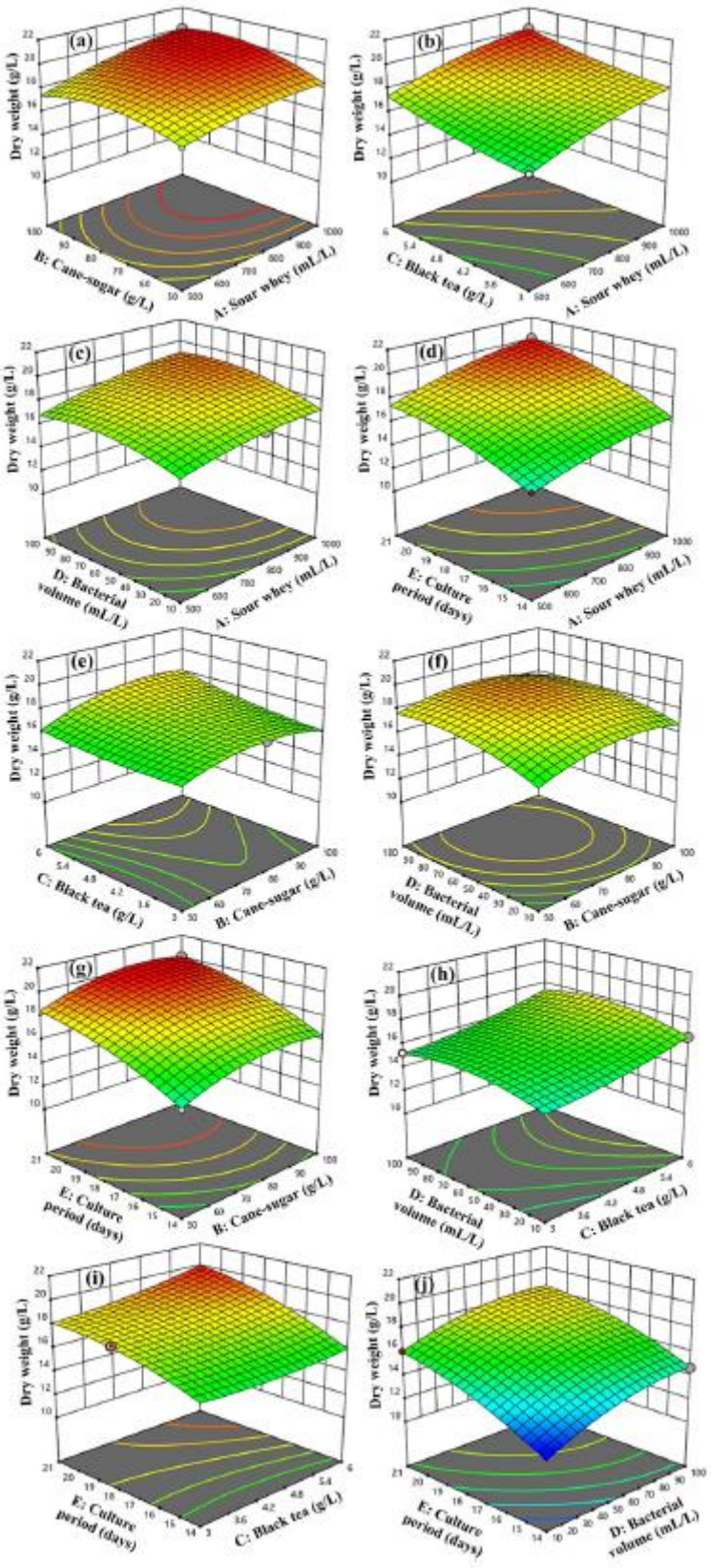


**FIGURE 2** Response surface diagram illustrating the two-factor interaction effect [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 5** Numerical optimization and validation tests

| Parameters | Goal | Experimental region | | Results | |
|---|---|---|---|---|---|
| | | Lower | Upper | Optimized | Validation |
| A: Sour whey | Maximized | 500 | 1000 | 1000 | 1000 |
| B: Cane sugar | In range | 50 | 100 | 87.23 | 87.23 |
| C: Black tea | In range | 3 | 6 | 6 | 6 |
| D: Bacteria volume | In range | 10 | 100 | 79.92 | 79.92 |
| E: Culture period | In range | 14 | 21 | 21 | 21 |
| Y: Dry weight | Maximized | 12.04 | 20.06 | 20.59 | 20.14 |

**TABLE 6** KBC dry weight and residual volume of the culture medium (ml) obtained in large containers with different cultured medium depths

| Samples | KBC dry weight (g) | KBC yield (g/L) | Residual volume of culture medium (ml) |
|---|---|---|---|
| KBC-0.5 cm | 12.68 ± 0.36 | 70.43 ± 2.00 | 72.33 ± 8.74 |
| KBC-1 cm | 19.01 ± 1.16 | 52.80 ± 3.22 | 226.67 ± 24.66 |
| KBC-2 cm | 22.39 ± 1.37 | 31.10 ± 1.90 | 566.67 ± 35.19 |
| KBC-3 cm | 22.41 ± 1.53 | 20.75 ± 1.42 | 916.67 ± 30.55 |
| HS-0.5 cm | 9.52 ± 0.86 | 52.87 ± 4.78 | 94.33 ± 7.23 |
| HS-1 cm | 10.74 ± 1.17 | 29.84 ± 3.25 | 271.67 ± 22.55 |
| HS-2 cm | 13.36 ± 0.41 | 18.55 ± 0.58 | 616.67 ± 41.63 |
| HS-3 cm | 13.28 ± 1.08 | 12.30 ± 1.00 | 976.67 ± 25.17 |

*Abbreviations: HS, Hestrin and Schramm; KBC, kombucha-derived bacterial cellulose.*

## 3 RESULTS AND DISCUSSION

### *3.1 Experimental design and statistical analysis*

The optimization results of KBC production using Design-Expert V11 software are summarized in **Table 3**. All independent variables have been expressed as highly effective, with a significant effect on the dry weight of KBC membranes compared to standard HS medium. Furthermore, at the end of the experiment time, the pH of the culture medium was maintained in the range of 4.4-4.9, still within the range of optimum pH (4-7) for cellulose production by Komagatacibacter xylinus.[13,27] The combined effect of the factors based on the design model is represented by the quadratic polynomial Equation (3).

$$\begin{aligned} Y = {} & 18.05 + 0.628A + 0.2829B + 0.1941C + 0.9095D \\ & + 1.39E + 0.378AB - 0.0537AC + 0.0219AD \\ & + 0.1977AE + 0.3894BC - 0.2219BD - 0.0026BE \\ & + 0.0456CD + 0.4261CE - 0.1593DE - 0.4127A2 \\ & - 0.9740B2 + 0.2851C2 - 0.8351D2 - 0.6292E2. \end{aligned} \quad (3)$$

where, *Y* is the dry weight of KBC membranes (g/L); A, B, C, D, E are the coding forms of the factor's sour whey, cane sugar, black tea, bacteria volume, and culture period, respectively, the coefficients AB, AC, AD, AE, BC, BD, BE, CD, CE, and DE are the form showing the interactions between the factors, and $A^2$, $B^2$, $C^2$, $D^2$, and $E^2$ are the forms of the quadratic effect. The (+) sign shows a positive or synergistic effect, while the (-) shows a negative or antagonistic effect.

The model statistical significance was determined by ANOVA analysis (**Table 4**) and indicated that probability *p* (<0.0001) was very small, with the determination coefficients $R^2$ and adjusted $R^2$ calculated as 99.86 and 99.45% respectively. This shows that the reliability of the model is high. The closer the value of $R^2$ is to 1.00, the more significant is the model and the better the predicted results. Close agreement (<0.2) between adjusted $R^2$ and predicted $R^2$ was observed demonstrating the suitability of the regression model in determining the optimal cellulose yield. In addition, the Adeq Precision (65.57) value was greater than 4, indicating that this model can be used to navigate the design space. On the other hand, the linear graph of the predicted response compared to actual (**Figure 1**) depicts that the data points are scattered along the diagonal, proving the model is satisfactory in the range of investigated parameters.

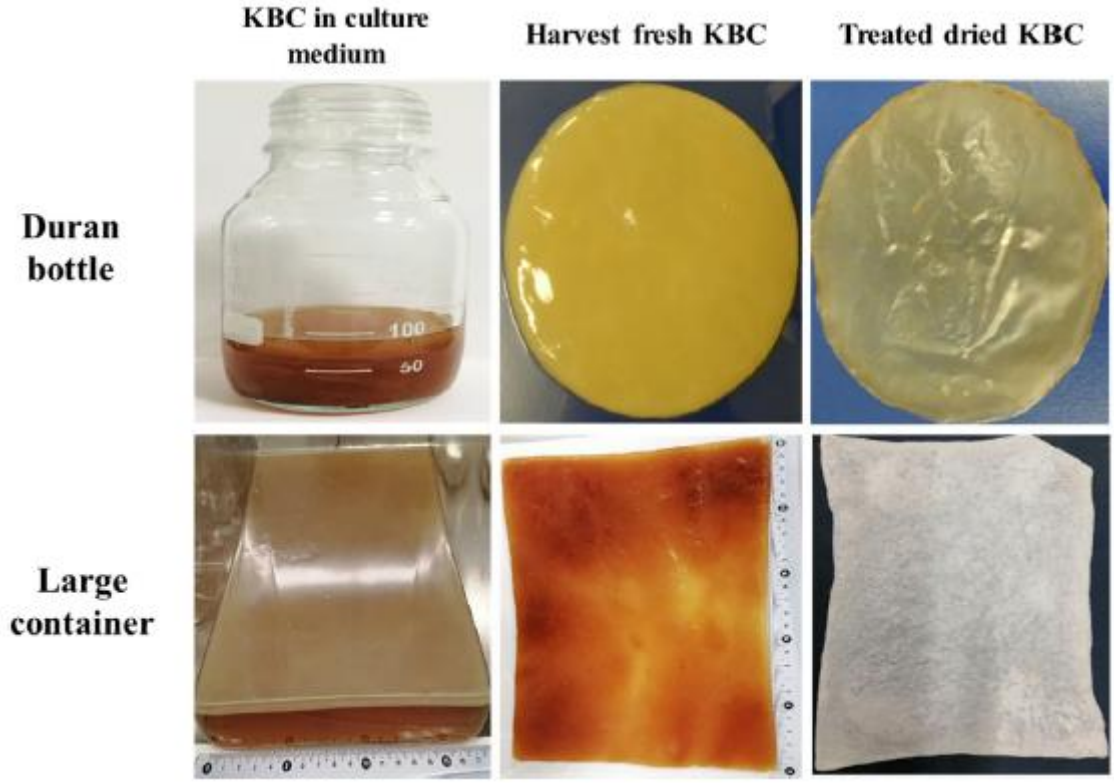


**FIGURE 3** Images of KBC membranes cultured at optimal conditions, harvested fresh KBC, and treated dried KBC. KBC, kombucha-derived bacterial cellulose [Color figure can be viewed at wileyonlinelibrary.com]

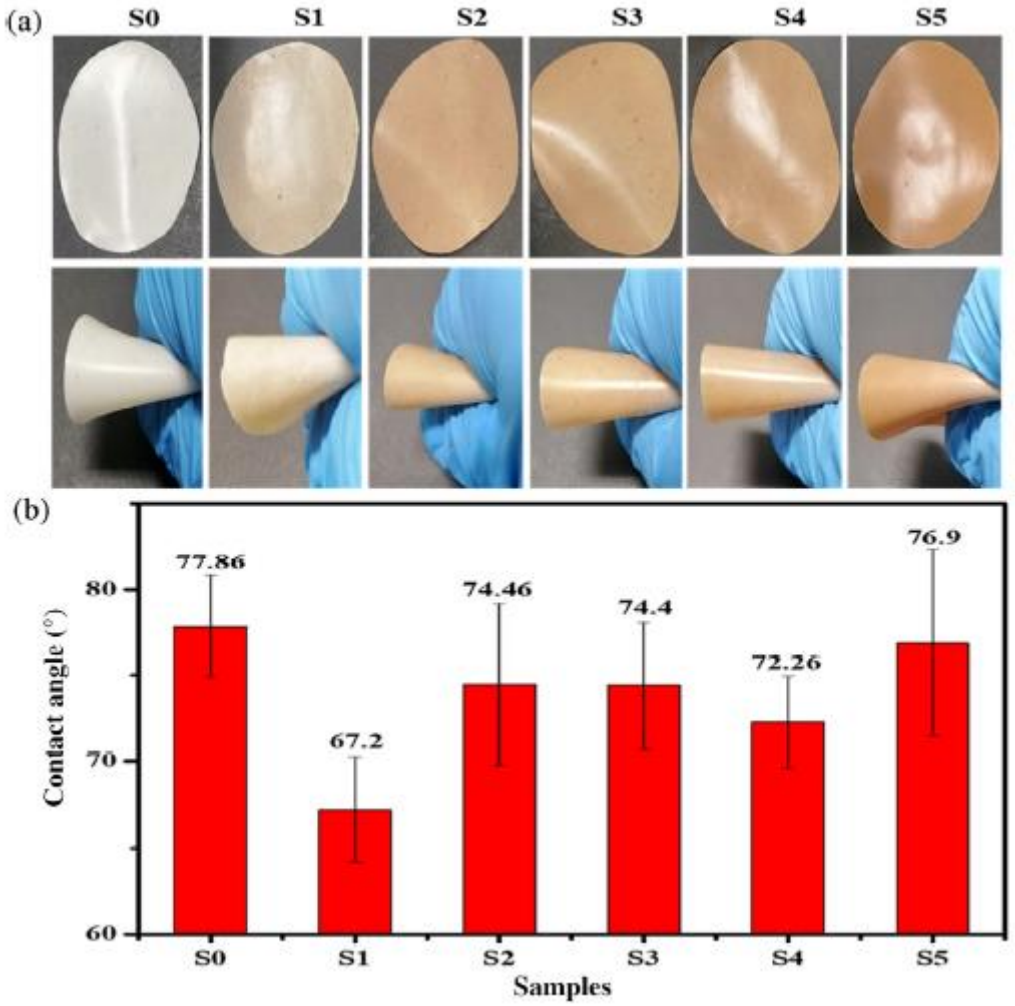


**FIGURE 4** (a) Physical appearance of biocomposites and (b) contact angle [Color figure can be viewed at wileyonlinelibrary.com]

*3.2 Numerical optimization and validation of model*

The 3D response surface plots (**Figure 2**) illustrates the combined effects of each pair of the independent variables on the response toward determining their optimal value. It can be observed that all five surveyed parameters had a significant effect on cellulose membranes dry weight. Considering the two factors cane sugar and bacteria volume, their values increased, while cellulose membrane dry weight increases followed by a decrease (**Figure 2**a,c,f).

This was attributed to the availability of carbon and nitrogen ratio (C/N) as well as the aerobic nature of *Komagatacibacter xylinus*. That is, if the concentration of some nutrients is in excess, they will create more by-products during metabolism, which inhibit the growth of major substrates, enhance cellulose production.[51] During growth, in the static culture mediums, cellulose membranes are formed on the surface of the culture medium (liquid-air interface), leading to limited oxygen intrusion. Therefore, if the bacterial cell density is high, this renders the cells "oxygen-starved" and unable to initiate growth, resulting in a decrease in cellulose production.[51,54,55] Meanwhile, the values of sour whey, black tea, and culture period increased, with increasing cellulose membrane dry weight will increase (**Figure 2**b,d,j).

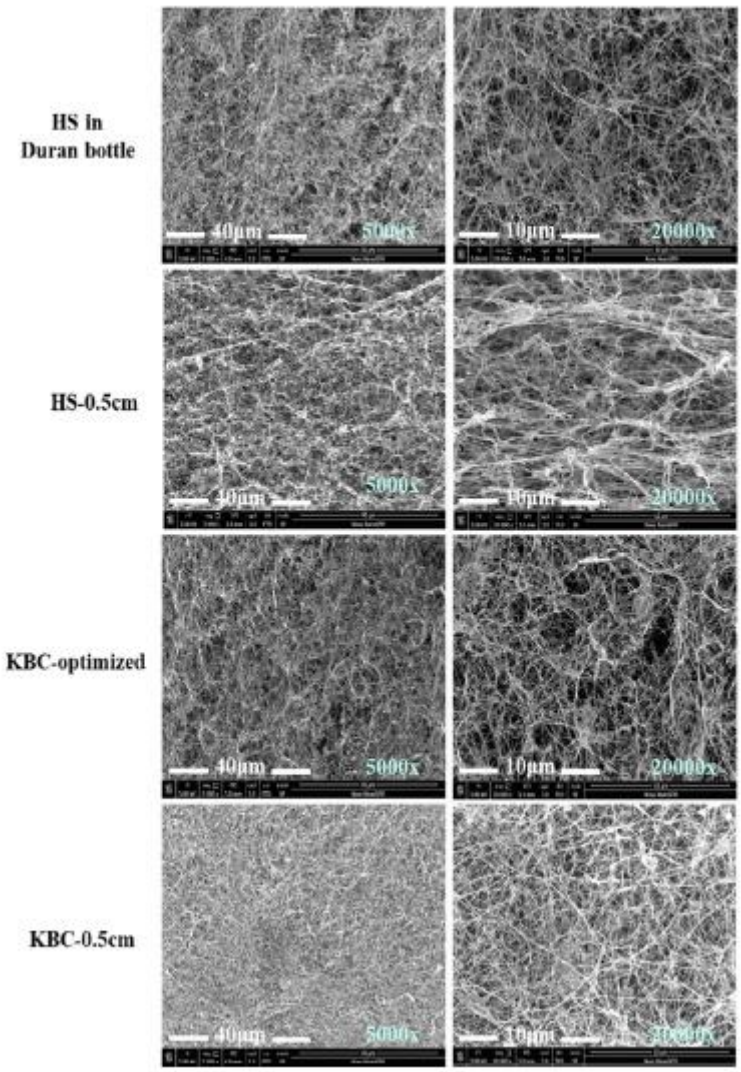


**FIGURE 5** SEM micrographs of obtained cellulose membranes. SEM, scanning electron microscope [Color figure can be viewed at wileyonlinelibrary.com]

Parallel to the obtained results, the model also proposed the best value for cellulose membrane dry weight shown as **Table 5**. A triplicate fermentation experiment using these optimization conditions was then conducted and an average dry weight of 20.14 ± 0.62 g/L was determined. This result depicted our model to be satisfactory in the group of highyield BC production (18.5-23 g/L), similar to the results obtained in previous reports by Raiszadeh-Jahromi et al., Bagewadi et al., Bekatorou et al., and Salari et al.[20,31,33,34]

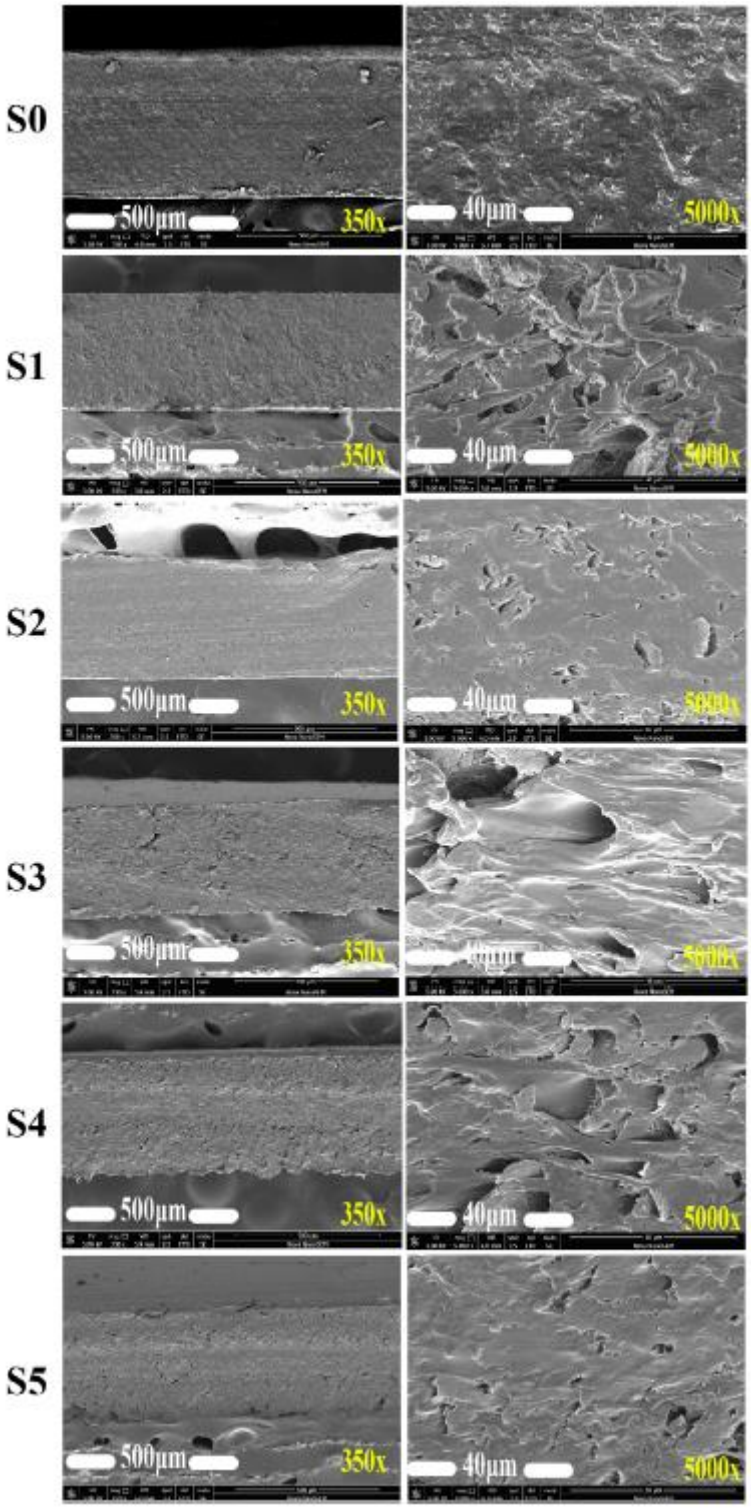


**FIGURE 6** Cross-sectional SEM micrographs of the prepared biocomposites. SEM, scanning electron microscope [Color figure can be viewed at wileyonlinelibrary.com]

*3.3 Determination of cultured medium depth in large containers*

**Table 6** and **Figure 3** displays the dry weight biomass and physical appearance KBC, BC by applying the optimal conditions for culturing in Duran bottles and large containers (surface area = 425 $cm^2$) with almost homologous cell density.

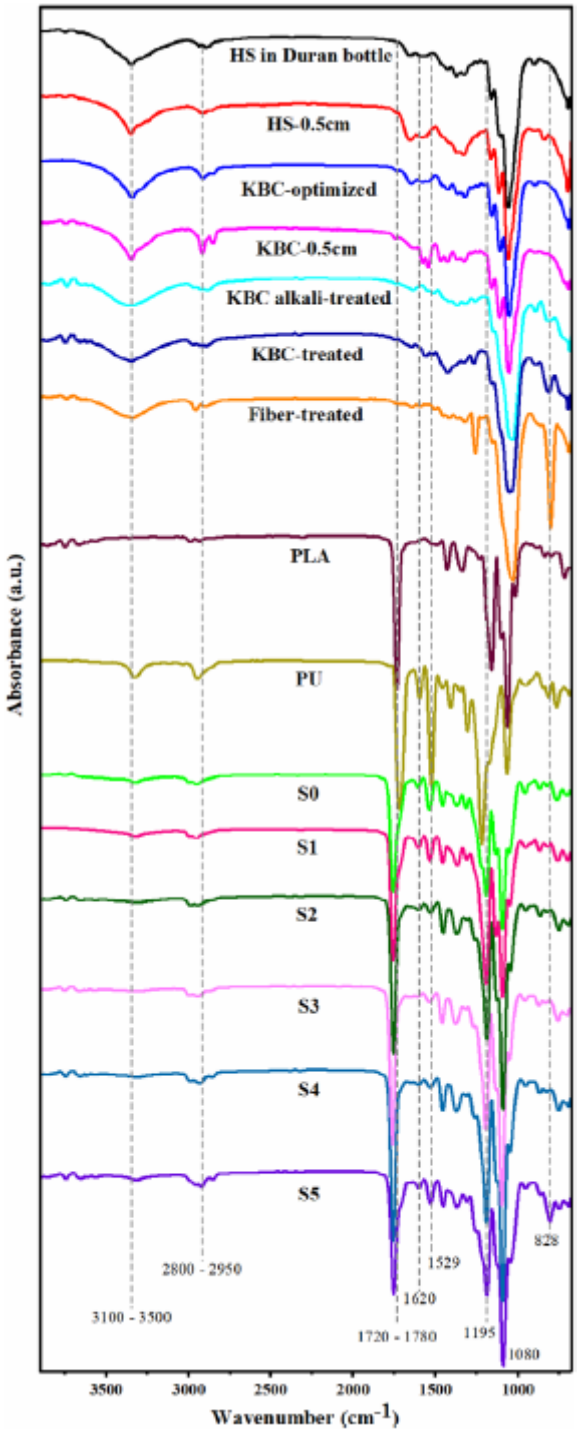


**FIGURE 7** FTIR spectra of prepared cellulose membranes and biocomposites [Color figure can be viewed at wileyonlinelibrary.com]

The cellulose yield continued to show high levels, especially with the dramatic increase in the experiments on standard HS medium in large scale. This can be attributed to the concentration of Komagatacibacter xylinus located in the air/liquid interface in the large containers that are hugely outnumbered compared to the same position in Duran bottle. Typically, the bacteria numbers have been readily exposed to unrestricted oxygen sources at the air/liquid surface for a longer time before being hindered by grown the cellulose membrane itself. Thus, cell respiration progressed more intensely, which in term enhance growth and cellulose biosynthesis.[40,51,54,55]

Considering the scale of one liter of the medium, the investigated fermentation batch efficiency achieved the highest at a cultured medium depth of 0.5 cm, corresponding to 180 mL (KBC-0.5 cm) with the thickness of KBC membranes reaching 17 ± 0.03 mm. Specifically, the residual volume of the culture medium (also called suspension) was extremely low, with only 72.33 ± 8.74 ml. This suspension possessed the potentials to be reused as a seed source for the next fermentation batch with an insignificant difference in the efficiency compared to the new *Komagatacibacter xylinus* source that was prepared in Section 2.2. This is extremely useful for a process of continuous production and sustainable development, even, toward closing the non-emission BC producing cycle. However, it is necessary to have more indepth research in terms of the determination of the number of reusable times as well as the difficulties of balancing yield and the requirement of the wider fermentation area, which can cause manufacturing cost high.

### *3.4 Fabrication of KBC biocomposites (leather-like materials)*

The prepared biocomposite materials possessed glossy, smooth surface, non-fracture, and less stiff properties with increasing color intensity from white (S0 as control) to brown (**Figure 4**a). This change in color intensity was attributed to the amount of incorporated KBC and sisal fiber (**Table 2**). Their surface wettability evaluated via water contact angle (WCA) measurements depicted the effectiveness of material source treatment (**Figure 4**b). All four samples containing KBC and sisal fiber treated with DCDMS (S2, S3, S4, and S5) possessed water resistance property all showing contact angles greater than 70°. This can be attributed to the grafting of silane on KBC and sisal fiber as a coating to avoid the adsorption of water since the modified micro-particles helped to improve the hydrophobic properties of the samples.[56-59] In contrast, the hydrophilic nature of pristine KBC and alkali treatment process increased the interaction of free hydroxyl groups via hydrogen bondings between the bio-fillers and water molecules leading to an increase in the surface wettability of sample S1.[60]

### *3.5 Morphological analysis of cellulose membranes and biocomposites*

The morphological properties of produced KBC were evaluated and compared to BC obtained from standard HS medium in Duran bottle and large containers with volume depth of 0.5 cm by SEM. All cellulose samples revealed similar characteristic fibrous 3D lattice structures arranged in a randomized and tightly manner (**Figure 5**). Image processing results determined the diameter of KBC-optimized in the range of 34-159 nm, 36-107 nm for KBC-0.5 cm and 21-147 nm for the two standard HS medium, which is in close agreement with results reported by Salari et al., Bekatorou et al., Santoso et al.[20,34,39]

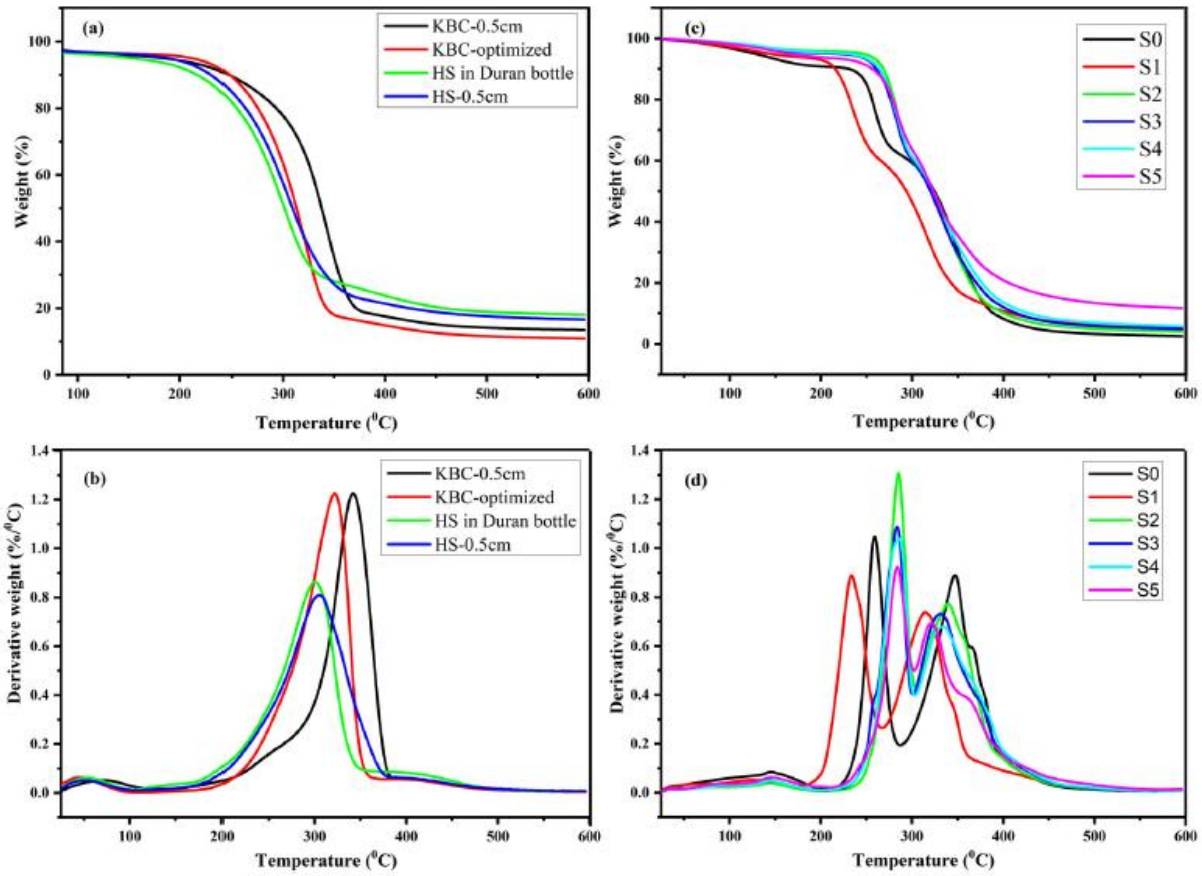


**FIGURE 8** Plots of (a and c) TGA and (b and d) DTG of prepared cellulose membranes and biocomposites. TGA, thermogravimetric analyzer [Color figure can be viewed at wileyonlinelibrary.com]

**Figure 6** illustrates the cross-sections of the prepared biocomposite materials. According to observed results, the samples showed tightly compacted structures without particle agglomeration, indicating homogenous blending between bio-fillers (KBC and sisal fibers) and the polymer matrix (PU and PLA). It is obvious that the main difference in SEM micrographs between the analyzed samples relaced to porosity and breathable properties. While the control sample (S0) possessed a smooth, nonporous structure, the other biocomposite samples demonstrated randomly arranged pores. The increase in the concentration of treated KBC with DCDMS (S2 < S3 < S4) was proportional to the increase in the density of the pores, but they were still far below that of alkali treated KBC sample (S1). In addition, the incorporation of high concentration of sisal fibers treated with DCDMS decreased the amount of PU and PLA used in S5. This recorded a difference in pores size and appearance. This difference in pore ratio plays a critical role in the properties revelation of the prepared composite materials, such as surface wettability, breathabil-ity, interfacial adhesion, or their mechanical strength.

### *3.6 FT-IR analysis of cellulose membranes and biocomposites*

FT-IR spectroscopy was used to detect the structural differences between the analyzed samples. Typical cellulose bonds and insignificant differences are seen in all KBC, BC, and sisal fiber samples. Parallel to that, all corresponding absorbance peaks of the bio-fillers (bio- fibers) and polymer matrix (PU, PLA) are present in all prepared biocomposites, as shown in **Figure 7**. The peaks at 3100-3500

(N—H and O—H stretching vibration), 2800-2950 (C—H asymmetric stratching in methyl, methylene, and methoxy groups), 1620 (O—H bending in cellulose as well as C—N and N—H stretching from PU), 1529 (amide II, N—H bending), 1195 (C—1O—C symmetric stretching), 1080 (C—N, C—C, and C O stretching) and 828 $cm^{-1}$ (Si—O—Si bending from DCDMS treated cellulose) were observed in the different prepared materials. The C=O bending band at 1720-1780 $cm^{-1}$ appeared with significant intensity relating to the presence of PU and PLA, which was observable for all prepared samples.[20,31,49,61,62]

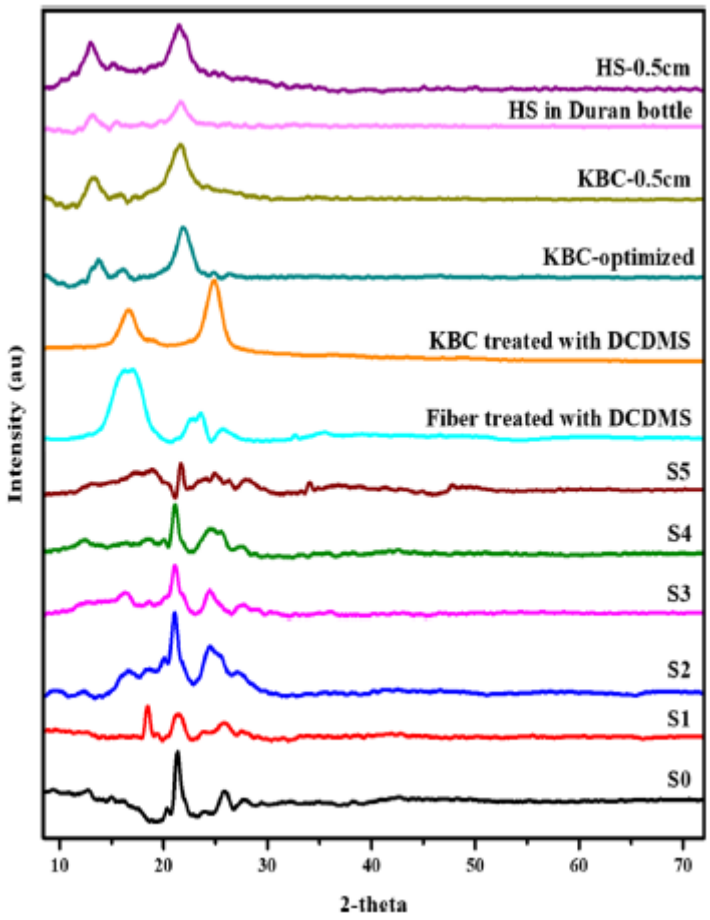


**FIGURE 9** XRD of cellulose membranes and prepared biocomposites. XRD, X-ray diffraction [Color figure can be viewed at wileyonlinelibrary.com]

### *3.7 Thermal analysis of cellulose membranes and biocomposites*

Thermal stability evaluation results of the investigated samples are shown in **Figure 8**. All samples exhibited gravitational thermal decomposition curves consisting of fairly similar degradation stages. The first degradation stages from 25 to 210° C for sample S1, 220° C for KBC and BC, and 240°C for other biocomposites are attributed to the slight weight decrease relating to water loss in the polymer matrix. This shows that the stability of all samples is higher than 200°C, demonstrating the great commercial application potential of these biopolymers. The second stage of degradation that occurred continues until 347 and 385°C is due to the decomposition of the polymer chains and network. Then, the maximum sample weight loss is observed at temperatures close to 400°C. Ultimately, the temperature between 400 and 600°C relates to carbon-char residues formation. However, small differences were also found between the samples. This may be attributed to the by-products of whey

and black tea in the cellulose biosynthesis fermentation medium or the amount of incorporated KBC and sisal fiber in the materials causing changes in the molecular weight, crystallinity, and orientation of the nanofibers. In addition, for KBC-0.5 cm and HS-0.5 cm, the cellulose fibers in these thicker samples appear to be tightly bonded, thus better thermal resistance to decomposition.[31,34,63]

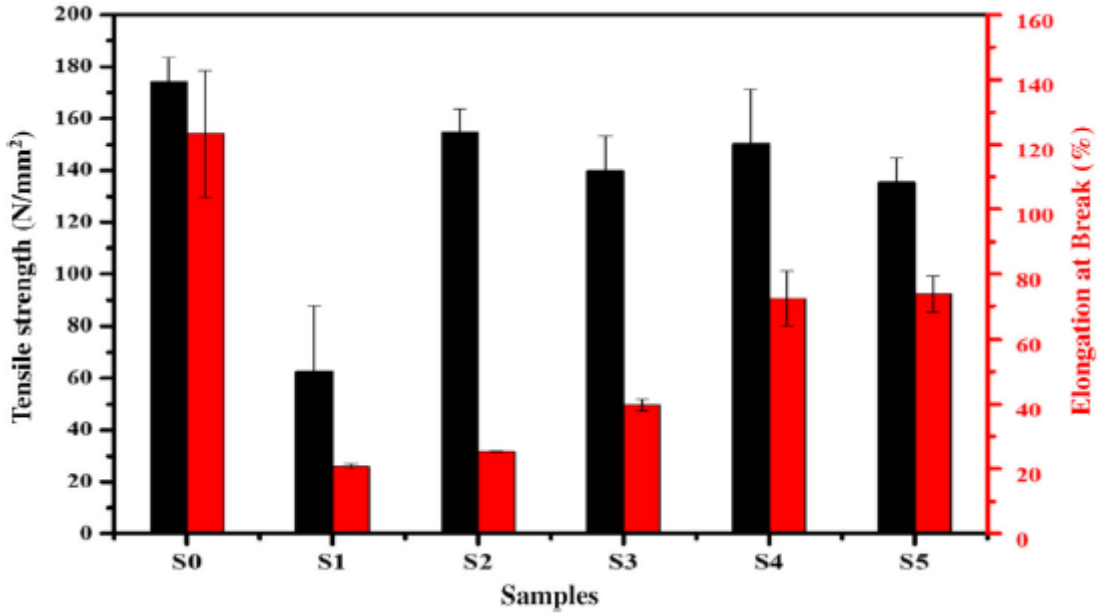


**FIGURE 10** Mechanical strength properties of the prepared biocomposites [Color figure can be viewed at wileyonlinelibrary.com]

*3.8 XRD analysis of cellulose membranes and biocomposites*

**Figure 9** displays the XRD analysis results to evaluate the crystal structure of the cellulose samples and biocomposite materials obtained. For cellulose membranes, the main vertices around 20 angles were determined at ≈14.7° and ≈22.6°, representing the cellulose structure type **Iα**, which corresponds to the diffraction plane (101) and the amorphous region (002) of pure cellulose in nature. The only difference between the obtained cellulose samples was the crystallinity percentages determined as 80.9, 81.5, 78.6, and 80.3%, for KBC-optimized, KBC-0.5 cm, BC obtained from standard HS medium in Duran bottle, and HS in a large container (HS-0.5 cm), respectively. For prepared biocomposite materials, the presence of two characteristic absorption peaks at 2θ angles around ≈21° and ≈26° of PU and PLA were found simultaneously in the XRD spectrum curve of the control sample (S0) as well as all remaining samples. In addition, the corresponding absorbance peaks of KBC alkali-treated or KBC and sisal fiber treated with DCDMS have also observed in the biocomposite contained them. The high crystallinity value determined for KBC-optimized and KBC-0.5 cm was possibly due to higher cultured medium pH, which led to the easy formation of hydrogen bonds between intermolecular or intermolecular groups in the bacteria cells. In addition, this may be attributed to the thicker of cellulose membranes obtained, causing restrictive bacterial movement, and ultimately creating cellulose molecule packing in a more orderly fashion.[39,49]

*3.9 Mechanical strength properties of the biocomposites*

As can be seen from **Figure 10**, there was a significant difference in the mechanical properties between the S1 and the remaining biocomposites samples. This is attributed to the difference in the degree of adhesion between the bio-fillers and the polymer matrix that constituted the material. Water removal treatment with DCDMS altered the hydrophilic properties of KBC-treated and fiber-treated, making the fibers to be more hydrophobic, therefore, increasing cohesion, and compatibility between the bio-fillers with polymer matrix, creating a more stable three-dimensional structure with improved toughness and flexibility. Specifically, their tensile strength and elongation at break recorded achieved impressive results, in the range of 135.61 ± 9.15 to 154.89 ± 9.09 N/mm$^2$ and 31.06 ± 0.32 to 92.33 ± 6.91%, much higher than of S1 sample (62.63 ± 24.97 N/mm$^2$ and 26.00 ± 1.02%).

In addition, the increase in the concentration of treated KBC with DCDMS resulted in an increase in elongation at break values of the obtained biocomposites (S4 > S3 > S2). This phenomenon was due to the ability of cellulose to form good bonding interactions, whereby, increasing the amount of KBC, increases the surface interactions, leading to an increase in the stress transfer efficiency of the interface, and thus, mechanical durability is enhanced.[61] However, the dominance in the mechanical strength of S0 (control sample) in the mechanical strength also showed the importance of PU for the structural stability of prepared blend bio-composites.[64] It is necessary for further studies to clarify the optimized concentration of KBC-treated combine with PU and PLA or other eco-friendly polymers, toward the fabrication of a suitable leather substitute material that can greatly respond to the expectations for safety, esthetics, properties, function, and also satisfy the consumer's requirements in ethics and social self-awareness.

## 4 CONCLUSION

The basic stages in the progression of transforming waste into usable materials or wealth have achieved encouraging initial results. A streamlined fermentation batch that removed unnecessary requirements (raw material pretreatment, pH calibration, shaking, stirring, aeration) shows high efficiency for an optimized process of cleaner production and sustainable development (outstanding yield, maximizing the amount of treated waste, and reducing new emissions). The obtained KBC properties depicted insignificant differences compared to BC from the standard HS medium, and have opened up countless application areas for these cellulose products. The final product, biocomposite materials, fabricated shows exemplary of specific application potential to be used in footwear, bags, and textiles with considerable flexibility and mechanical strength. Although the tensile strength values and elongation at break satisfied the requirements of the standard (normal footwear >10 N/mm$^2$), when compared to commercial PU-based leather materials, these prepared biocomposites will require continued improvement in vital features. Further research work such as treatment of the cellulose fibers to incorporate other bulky hydrophobic for better interaction with the polymer matrix and exploring the increase of fiber content in the blend composition is required. This may enhance mechanical integrity including; flexibility, texture, tear-resistance, breathability, thermal stability, and reduced wear for this sustainable design of nonwoven materials to meet the quality of good alternative with non-rising negative environmental effects in the footwear industry.

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