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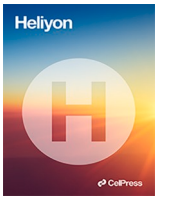

Research article

Bacterial cellulose biosynthesis: Optimization strategy using iranian nabat industry waste

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ABSTRACT

Bacterial cellulose (BC) is a biopolymer has found extensive applications across different fields due to its nanostructure and biomaterial performance. This study focused on optimizing yield of BC produced by *Komagataeibacter xylinus* CH1, isolated from kombucha SCOBY. The study aimed to use Nabat industry waste (NIW) as a cost-effective alternative carbon source for submerged fermentation. To optimize the fermentation criteria, the central composite design was used with the inoculation amount (1.5–4.5 % VV^{-1}), NIW (0–1%), and fermentation time (3–7 days) as independent variables. The impressive results indicated the yield was enhanced up to 45.543 gL^{-1} at 3.013 % VV^{-1} of inoculation, 0.516 % NIW, and 7 days of stirred fermentation. SEM, XRD, FTIR, and TGA were applied to evaluate the characteristics of freeze-dried BC, such as the three-dimensional, porous structure, crystalline peaks, amorphous haloes, and thermal stability. The physicochemical properties of BC including high moisture content (93.022 ± 0.472 %), water absorption rate (569.473 ± 3.739 %), water-holding capacity (1333.016 ± 3.680 %), porosity (166.247 ± 2.055 %), and low water activity (0.296 ± 0.030 %) were achieved. Rheological properties of BC suspensions showed that G′ dominated over G″, with tan δ values lower than 1. These characteristics indicate NIW and stirred fermentation conditions are a promising method for producing BC in high yield.

1. Introduction

Over the past few years, significant focus has been placed on the biosynthesis of bacterial cellulose (BC) because of its inherent advantages in lower density, higher crystallinity, greater water holding capacity, high mechanical strength of a web-like network structure, biodegradability, and more purity [1–5]. This kind of pure biocellulose comprises of glucose monomers linked by β(1–4) glycosidic bonds, with the molecular formula of $(C_6H_{10}O_5)n$ [6,7]. The cellulose pellicle is made up of fibrils that are randomly assembled and are less than 100 nm wide [7,8]. BC has become a preferred substitute for plant cellulose in a wide range of industrial sectors, such as biomedical materials, wastewater treatment, conductive materials, paper, cosmetics, biomedicine, and functional foods. Recent research has also explored BC applications in food industry, where it can be used as an additive to enhance food rheology,

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act as a thickening, stabilizing, or gelling agent [3,4,7].

BC is produced by certain and special strains of bacteria, with most cellulose-producing bacteria belonging to the genera *Gluconacetobacter* and *Komagataeibacter*, as detailed in recent reviews [9,10]. *Komagataeibacter xylinus*, that previously known as *Gluconacetobacter xylinus* or *Acetobacter xylinus,* has been extensively studied for its high and extended amount of pure BC production. The morphology, crystal structure, and physicochemical properties of the produced BC can be altered by different strains and fermentation conditions [3–5].

The industrial production and utilization of BC for commercial purposes have been constrained by its yield and production costs resulting from large-scale substrate shortage. Approximately 30 % of the fermentation cost of BC is attributed to the culture medium. Therefore, it is crucial to utilize cost-effective carbon sources and implement shorter fermentation methods to achieve high-yield BC production as new strategies [3,7]. While glucose has been the favored carbon source for BC biosynthesis, agricultural waste and by-products; such as pecan shells, sugar cane syrup, pineapple pomace, sweet sorghum, winery waste, olive oil mill waste water, miscanthus, sweet potato residues, and agricultural lotus rhizome; have been explored as alternatives due to their affordability and availability [2–4,11–15].

One of the potential sources of carbon for the fermentation process of BC production is the waste obtained from Iranian Nabat factories. Nabat, the Persian sweetener for hot drinks and an old generation of modern candies, is widely consumed in Iran due to its health benefits. Iranians have considered Nabat as a traditional medicine for a long time. Razavi Khorasan province (Mashhad) is considered the largest Nabat production area, producing over 210,000 tons per year. Nabat is created through the crystallization of a saturated solution of sucrose via a physical mechanism dependent on time and temperature. Nabat production is associated with about 25 % of waste. Nabat industry waste (NIW) is separated from the cooled super-saturated sugar solution that did not crystallize during Nabat formation. It contains 25 % glucose, 25 % fructose, and 50 % sucrose [16,17].

In addition to carbon sources, the fermentation method is crucial in BC production. Static and stirred fermentation conditions are two basic techniques used for BC production. BC yield under the static method is lower than the stirred method and requires a longer cultivation time and extensive space [4,18]. Different forms of nanocellulose are produced under these two kinds of conditions. Under static fermentation, BC is created in sheet, membrane, or film form by three-dimensional interconnected reticular pellicles. On the other hand, fermentation under the stirred method leads to the production of BC in the form of small sphere-like granules and irregularly shaped pellets. Most research has focused on static fermentation, with fewer studies on the stirred technique [19–21].

Following mentioned above, this study aimed to investigate the impact of bacteria inoculation percentage, fermentation time and the use of NIW; as a new alternative carbon source, on the optimization of BC yield produced by *Komagataeibacter xylinus* CH1 strain (isolated from kombucha SCOBY); under continuous stirred fermentation conditions using the response surface method (RSM). Moreover, various characteristics of the produced BC, including purity, physicochemical properties, morphology, rheology, and thermogravimetric properties, were examined. In this study we have used NIW, for BC biosynthesis for the first time.

2. Materials and methods

2.1. Materials

D-glucose, glycerol, yeast extract, peptone, citric acid ($C_6H_8O_7$), disodium phosphate (Na_2HPO_4), sodium hydroxide (NaOH), sodium citrate ($Na_3C_6H_5O_7$), and cellulase enzyme were acquired in their analytical grade from Merck Co. (Germany) and Sigma Co. (USA). Nabat industry waste (NIW) was obtained from Saharkhiz Nabat Industries Co. (Iran, Mashhad), containing: 25 % glucose, 25 % fructose, 50 % sucrose and Brix = 72 %.

2.2. Methods

2.2.1. Bacterial cellulose production

In this research, *Komagataeibacter xylinus* CH1 strain, isolated from the kombucha SCOBY sample purchased from Joshua Tree Kombucha Co. (China), and identified by comparing the sequences obtained from the 16S rRNA gene with the sequences available in the NCBI database, was exploited to produce BC.

First, the *Komagataeibacter xylinus* CH1 strain was incubated in Hestrin-Schramm (HS) medium (containing 10 gL^{-1} D-glucose, 10 gL^{-1} glycerol, 5 gL^{-1} yeast extract, 5 gL^{-1} peptone, 2.7 gL^{-1} Na_2HPO_4, 1.15 gL^{-1} citric acid, pH = 6), at 30 °C for 72 h to produce a pre-culture, with a count of about 12 log $CFUmL^{-1}$.

For yield optimization, fermentation was carried out in 100 mL sterilized erlenmeyer flasks containing 30 mL of the main culture medium with consideration of variables. The flasks were incubated at 30 °C, at a speed of 120 rpm under continuous stirred fermentation conditions in a shaking incubator (HYSC, Korea). At the end of fermentation, BCs were harvested and used for next steps [19,22].

2.2.2. Experimental design for optimization

Optimizing BC yield depended on a Face-center design, with three independent variable factors, including days of fermentation (A), amount of NIW (B), and the percentage of inoculation (C), carried out by response surface methodology (RSM) with six replicates of a central point (central composite design (CCD)), using Design-Expert software (Version 13). There were a total of 20 runs for optimizing the BC yield (Table 1) and a reduced cubic model using actual variables was fitted for the obtained response.

To evaluate the accuracy of the optimized model, R^2 (coefficients of determination), Adjusted-R^2, CV (coefficient of variation), P-

value and lack of fit were calculated.

2.2.3. BC purification

For purification, first, BC pellicles were washed several times and boiled in a 0.5 M aqueous solution of NaOH for 15 min using a bain-marie (Memmert, Germany). Then, BC was rewashed until the pH became neutral and the residue of bacterial cells and culture medium was removed. Next, the BC was soaked in deionized water overnight until became cream-like color. Finally, it was dried by a freeze dryer at −60 °C for 48 h (Operon, Korea) [19,23].

2.2.4. Determination of the yield of BC

After freeze-drying, the weight of dried BC per liter of culture medium applied in the fermentation process was reported as yield (gL^{-1}) [19,23].

2.2.5. Evaluation of BC characterization

2.2.5.1. BC hydrolysis by cellulase. This test was applied to verify the purity of the produced bacterial cellulose. The BC pellicle was transferred to an erlenmeyer flask containing 50 mL of 2.5 % cellulase enzyme in 1 M citrate buffer (29.5 mL of 1 M sodium citrate and 20.5 mL of 1 M citric acid). To complete the enzymatic hydrolysis reaction, the flask was incubated in a 37 °C incubator for 2 h. The appearance of clear liquid without any precipitation confirms the purity of the BC [24].

2.2.5.2. Moisture content. The moisture content of BC gels was determined based on the weight loss of BC gels placed in an oven (105 °C) (Memmert, Germany). The samples were dried until they reached equilibrium and calculated by Eq. (1) [19,23].

$$\text{Moisture content } (\%) = \left[\frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}}\right] \times 100 \tag{1}$$

2.2.5.3. Water-holding capacity (WHC) and water absorption rate (WAR). The weight of purified BC was measured before (wet weight) and after (dry weight) freeze-drying, and also after soaking dried BC in double distilled water for rewetting (rewet weight) at the room temperature for 48 h. WHC and WAR were calculated by Eqs. (2) and (3) respectively [19,25].

$$\text{WHC } (\%) = \left[\frac{(\text{wet weight} - \text{dry weight})}{\text{dry weight}}\right] \times 100 \tag{2}$$

$$\text{WAR } (\%) = \left[\frac{(\text{rewet weight} - \text{dry weight})}{\text{dry weight}}\right] \times 100 \tag{3}$$

2.2.5.4. Porosity. First, after BC production, the weight of wet BC measured as “wet weight”. Then the wet BC dried by freeze-drying and the weight of dried BC calculated as “dry weight”. At the next stage, a certain amount of dried BC was soaked in deionized water at the room temperature for 24 h and the weight of BC was measured as “rewet weight”. Finally, the porosity calculated using Eq. (4) [19].

$$\text{Porosity } (\%) = \left[\frac{(\text{wet weight} - \text{dry weight})}{(\text{wet weight - rewet weight })}\right] \times 100 \tag{4}$$

2.2.5.5. Water activity (a_w). The water activity of dried BC was operated at 25 °C using the NOVASINA a_w measurement (Labmaster, Switzerland) [26].

2.2.5.6. Determination of bulk and tapped density. The ratio of a specified weight of the dried BC sample to volume that is occupied was calculated as bulk density using Eq. (5). To measure the tapped density, a certain amount of powder samples gently was loaded into a graduated clean and dry cylinder and tapped on the cylinder 200 times. The final volume of BC was considered for calculation of tapped density by Eq. (6) [27].

$$\rho b \ (g / ml) = \frac{m}{v} \tag{5}$$

$$\rho t \ (g / ml) = \frac{m}{v} \tag{6}$$

2.2.5.7. Thermogravimetric analysis (TGA). Dried BC was ground and transferred into a 100-mesh sieve (0.149 mm). Analyses were performed by about 1 mg sample in 150 mm aluminum pans under a dynamic nitrogen atmosphere (flow rate of 20.0 mL min^{-1}), temperature variety from 30 °C to 1000 °C, at the heating rate of 10 °C min^{-1}. TGA curves were recorded by SDT Q600 V20.9 Build 20 (TA Instruments Inc, USA) [19,28].

Table 1
Experimental design matrix for parameter levels.

Run	Coded level			Actual level			Run	Coded level			Actual level		
	A	B	C	Fermentation (day)	amount of NIW (%)	inoculation (%)		A	B	C	Fermentation (day)	amount of NIW (%)	inoculation (%)
1	−1	−1	+1	3	0	4.5	11	0	0	0	5	0.5	3
2	+1	0	0	7	0.5	3	12	−1	−1	−1	3	0	1.5
3	−1	+1	+1	3	1	4.5	13	+1	+1	−1	7	1	1.5
4	0	0	0	5	0.5	3	14	0	0	0	5	0.5	3
5	−1	0	0	3	0.5	3	15	+1	−1	−1	7	0	1.5
6	0	0	0	5	0.5	3	16	+1	−1	+1	7	0	4.5
7	0	0	0	5	0.5	3	17	0	0	−1	5	0.5	1.5
8	0	+1	0	5	1	3	18	0	0	+1	5	0.5	4.5
9	−1	+1	−1	3	1	1.5	19	0	−1	0	5	0	3
10	+1	+1	+1	7	1	4.5	20	0	0	0	5	0.5	3

A: days of fermentation, B: amount of NIW, C: percentage of inoculation.

2.2.5.8. Fourier transforms infrared spectroscopy (FTIR). Dried BC was ground and mixed with potassium bromide (KBr) powder; 20 mg of the combination was hard-pressed into a tablet. The FTIR spectrum was investigated using a Thermo Nicolet AVATAR 370 (USA) in the transmittance mode at the wavenumber range of 4000 to 400 cm^{-1} with the resolution of 4 cm^{-1} [19].

2.2.5.9. X-ray diffraction (XRD). Ground dried BC was applied for x-ray diffractometry (XRD) analysis. The diffractograms were recorded at the room temperature, using Ni-filtered Cu (Copper), and X-ray radiation ($\lambda = 1.54056$ A°). The operating situations were 40 kV and also 40 mA. Data were scanned in reflection mode in the 10–60° 2θ range with a step of 0.05° 2θ intervals. The scans continued at 1 s per step (Model Explorer 01, GNR, Italy). HIGH SCORE PLUS software was used to process the diffraction pattern and calculate the crystallinity, 2θ peak positions, and d-spacing of dried BC [19,29]. The degree of crystallinity (X_c), was calculated by the ratio of the crystalline region to the total area (crystalline (S_{cr}) and amorphous (S_a)) (S_a) by Eq. (7) [30], The crystal sizes of the BC sample were calculated by the Scherrer equation by Eq. (8) [30], The crystallinity index (CI) of the BC sample was calculated by the Segal method by Eq. (9) [31].

$$XC\,\% = \frac{S\,cr}{S\,cr + S\,a} \times 100 \tag{7}$$

$$D = \frac{K\,\lambda}{B\,COS\,\theta} \tag{8}$$

$$CI\,\% = \frac{I\,cr - I\,non\,cr}{I\,cr} \times 100 \tag{9}$$

2.2.5.10. Scanning electron microscopy (SEM). The morphology and fibrillar structure of dried BC was studied by SEM. A thin layer of BC sample was coated with gold by an ion sputter coater and observed at 20 kV, by a microscope (LEO, VP 1450, Germany) operated under 100X, 250X, 1000X, 2500X, 5000X, and 10000× magnification [32].

2.2.5.11. Color measurement of BC. Dried BC was ground and powdered for color measurement. The sample was poured onto a clear plate, placed in a box painted black tint to minimize background light, and scanned by a scanner (Canon LIDE 320, Vietnam). The scanned images were at that time analyzed by Image J software (version 4.1.3, USA). The basis of colorimetry was the measurement of L* (brightness/darkness), a* (redness/greenness), and b* (yellowness/blueness) indicators [33].

2.2.5.12. Rheological properties measurement. For evaluation the rheological behavior of dried BC, three different suspensions containing 1, 1.5, and 2 % (wv^{-1}) of dried BC powder were prepared in distilled water. The analysis was performed by a Physica MCR 302 SNB2492533 rheometer (Anton Paar GmbH, Stuttgart, Germany), using a parallel plate (PP25-SN55722, 25 mm in diameter, and a gap size of 1 mm) that was controlled by a normal force of 0.2 N with triplicate deliberations. The temperature for all measurements was kept constant at 25 °C with a Peltier system (Viscotherm VT2) with an accuracy of ±0.01 °C. To allow temperature equilibration, the samples were permitted to rest for 5 min before the start of the test [29]. For frequency sweep tests, initially, each sample was subjected to a dynamic strain sweep from 0.001 to 100 % and a frequency of 1 Hz to determine the linear viscoelastic region (LVR). Frequency sweep tests were made at an optimal strain of LVR 0.01 % with frequency between 0.01 and 100 Hz [29]. The parameters including storage modulus (G′), loss modulus (G″), complex modulus (G*), complex dynamic viscosity (η*), loss tangent (tan δ), yield strain (γL), yield stress (τy), flow point strain (γf), flow point stress (τf) and $G' = G'' = Gf$ were calculated.

Steady flow behavior tests were done at shear rates of 0.1–100 s^{-1} in 900 s and the data were fitted by Power Law model (Eq. (10)).

$$\tau = k\dot{\gamma}^{n} \tag{10}$$

Where τ is shear stress (Pa), k is the consistency coefficient (Pa s^n), $\dot{\gamma}$ is the shear rate (s^{-1}) and n is the flow behavior index (dimensionless).

Table 2
Yield optimization of freeze-dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions.

Run	Variables			Yield (gL^{-1})	Run	Variables			Yield (gL^{-1})
	Fermentation (day)	amount of NIW (%)	inoculation (%)			Fermentation (day)	amount of NIW (%)	inoculation (%)	
1	3	0	4.5	2.900	11	5	0.5	3	23.867
2	7	0.5	3	45.533	12	3	0	1.5	3.033
3	3	1	4.5	2.233	13	7	1	1.5	14.900
4	5	0.5	3	23.100	14	5	0.5	3	22.967
5	3	0.5	3	1.833	15	7	0	1.5	17.933
6	5	0.5	3	23.400	16	7	0	4.5	16.600
7	5	0.5	3	23.000	17	5	0.5	1.5	13.967
8	5	1	3	10.567	18	5	0.5	4.5	25.200
9	3	1	1.5	1.967	19	5	0	3	13.667
10	7	1	4.5	17.033	20	5	0.5	3	21.933

2.3. Statistical analysis

The optimization of BC was examined by Response Surface Methodology (RSM), Central Composite Design using Design Expert software version 13. The physicochemical properties of BC were conducted in three replicates. The mean and standard deviation of the data were calculated by Minitab software version 19.

3. Results and discussion

3.1. Yield of BC

Optimized BC yield values are presented in Table 2. Table 3 shows the ANOVA analysis and regression values of independent variables for BC yield. Based on the yield % results, the non-linear quadratic multivariate models were representative models that could predict the values of independent variables, interactions, and quadratic effects (Eq. (11)). The calculated R^2 and CV % values were 0.9985 and 4.19 for the BC yield.

$$Y = +39.64105 - 23.33795\,A - 96.66751\,B - 10.67540\,C + 24.46650\,AB + 9.19425\,AC + 0.644167\,BC + 2.68296\,A^2 + 104.78157\,B^2 - 1.49291\,C^2 + 0.504187\,A^2B - 0.916646\,A^2C - 29.61675\,AB^2 \quad (11)$$

(A: days of fermentation, B: amount of NIW, C: percentage of inoculation)

As shown in Table 3, the linear and quadratic terms of A, B, C, B^2, C^2, A^2C, and AB^2 had significant effects ($p < 0.05$) on the BC efficiency.

As shown in Fig. 1(a) and (b), substitution of Nabat industry waste up to 0.5 % resulted in an increase in the production of BC, however higher amounts of waste led to a decrease in the yield. The changes in the amount of inoculation (from 1.5 min to 4.5 %) also had similar effects on the BC yield. Meanwhile, increasing the fermentation time from 3 to 7 days caused a sharp increase in the value of BC yield.

Based on the data extracted from the analysis, the optimized conditions for BC production by *Komagataeibacter xylinus* CH1 were determined as 3.013 % VV^{-1} percentage of inoculation, 0.516 % amount of NIW, and 7 days of fermentation under constant stirred fermentation conditions (pH = 6, 30 °C, 120 rpm). The optimization yield was enhanced up to 45.543 gL^{-1}.

Researchers' results showed that the yield of BC was notably influenced by the type of carbon source [23]. compared various carbon sources (glucose, fructose, glycerol, sucrose, mannitol, starch, and whey) for BC manufacture by *Gluconacetobacter xylinus*. According to their results, glycerol produced the highest yield (1.9 gL^{-1}), followed by glucose and fructose. In another study, it is reported that *Komagataeibacter xylinus* K1011 and *Komagataeibacter xylinus* K975 had the highest BC yield, 4.69 gL^{-1} and 3.54 gL^{-1} respectively, when fructose was utilized as the carbon source instead of glucose, under stirred fermentation conditions [34]. Based on the research conducted by Bae, Sugano, & Shoda (2004), the yield of BC produced by *Gluconacetobacter xylinus* (BPR2001) under agitated conditions and with fructose as the carbon source was recorded at 14.10 g/L^{-1}.The effect of the simultaneous use of glucose and fructosein BC production was investigated by Ref. [35]. The results showed that using a combination of two monosaccharides has a better effect on the BC production compared to using each one alone. Also, in the ratio of 1–3 glucose to fructose, the highest efficiency of BC was obtained. Also, based on the research of [36], in stirred conditions, *Acetobacter xylinus* sp. A9 produced 15.2 gL^{-1} of BC by

Table 3
ANOVA analysis in reduced cubic model of yield optimization of freeze-dried BC.

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	2224.17	12	185.35	398.05	<0.0001	significant
A- days of fermentation	954.85	1	954.85	2050.60	<0.0001	
B- amount of NIW	4.80	1	4.80	10.32	0.0148	
C- percentage of inoculation	63.09	1	63.09	135.49	<0.0001	
AB	0.0940	1	0.0940	0.2018	0.6669	
AC	0.0556	1	0.0556	0.1194	0.7398	
BC	1.87	1	1.87	4.01	0.0853	
A^2	1.51	1	1.51	3.24	0.1150	
B^2	322.28	1	322.28	692.12	<0.0001	
C^2	31.03	1	31.03	66.64	<0.0001	
A^2B	1.63	1	1.63	3.49	0.1038	
A^2C	48.40	1	48.40	103.94	<0.0001	
AB^2	350.86	1	350.86	753.50	<0.0001	
Residual	3.26	7	0.4656			
Lack of Fit	1.21	2	0.6051	1.48	0.3135	not significant
Pure Error	2.05	5	0.4099			
Cor Total	2227.43	19				
R^2						0.9985
Adjusted R^2						0.9960
Std. Dev.						0.6824
C.V. %						4.19

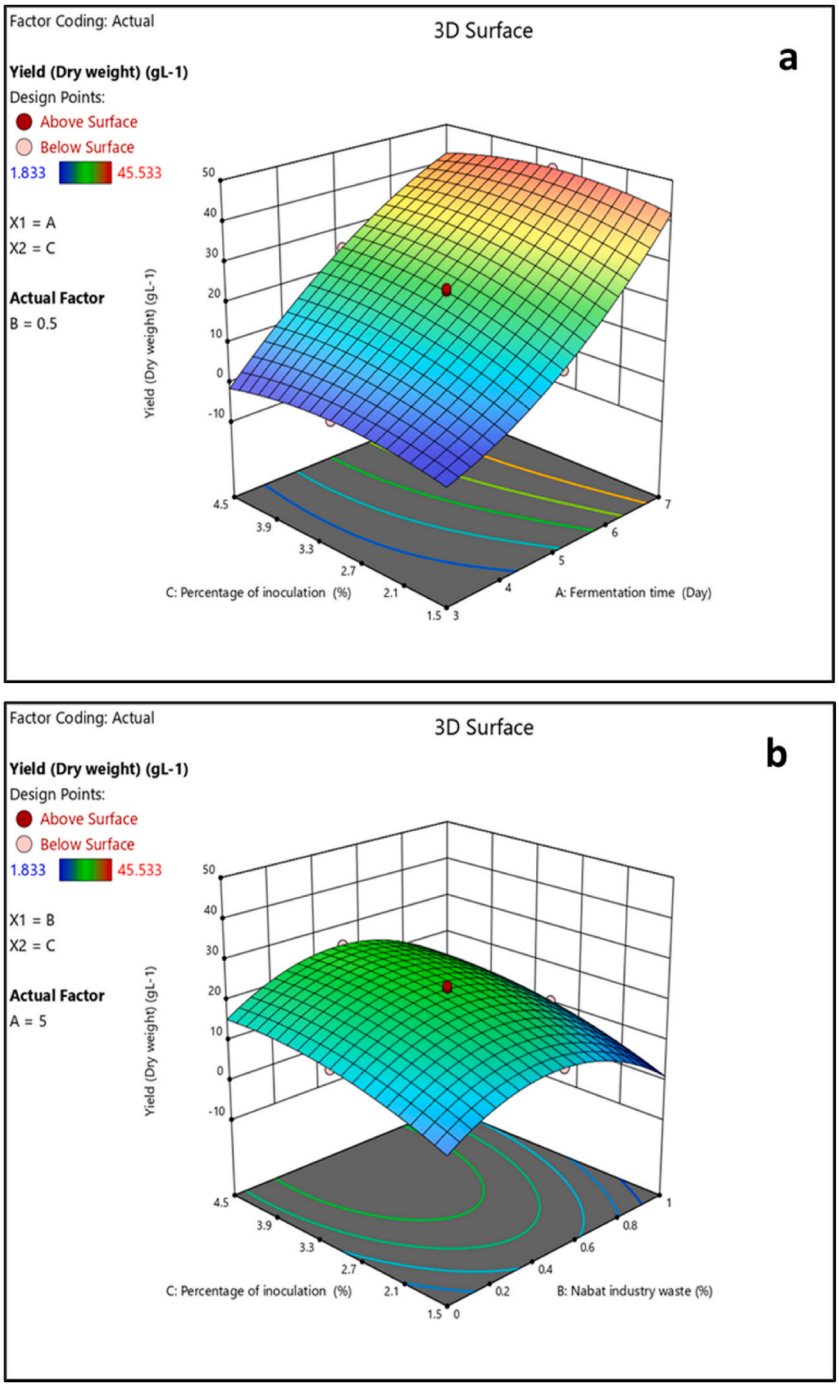

Fig. 1. (a) Effect of days of fermentation and percentage of inoculation, (b) Effect of amount of NIW and percentage of inoculation on the yield of dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions, ($p > 0.05$).

using glucose and ethanol. The BC yield was influenced by the strains, the amount of inoculation, and fermentation conditions. The quantity of BC produced by initial concentration of 8 % v/v inoculum of *Komagataeibacter* sp. Nov. CGMCC 17276, at 160 rpm, and 4 days of culture, achieved 5.24 gL^{-1} when 4 % glycerol was used [37]. In another study *Komagataeibacter xylinus K*975, and *Komagataeibacter xylinus* K1011 produced 3.54 and 4.69 gL^{-1} of BC, respectively, at 150 rpm [34]. *Gluconacetobacter xylinus* ZHCJ618 produced 8.31 gL^{-1} BCE under shaking conditions [38]. Also, *Komagataeibacter xylinus* strains were compared in BC production under the stirred method. The BC yield varied between 2.9 and 3.4 gL^{-1} depending on the species [39].

3.2. Characterization of BC

The characteristics of dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation including water absorption rate, water-holding capacity, water activity, porosity, bulk and tapped density and the moisture content of BC gel, are shown in Table 4. The results indicated that the water absorption rate and water holding capacity of dried BC under stirred fermentation conditions were high. The high moisture content of BC gel, WHC and WAR could be explained by the BC structure, a bio-polymer of β-d-glucopyranose by (1 → 4) glycosidic bonds with many hydroxyl groups, making it such a unique hydrophilic material. The capillary force of the BC network, combined with its membrane-like shape and exceptional water-holding capacity, makes it well-suited for water-binding food additives and biomaterials [19,40,41].

The porosity of BC was 166.247 ± 2.055, as shown in Table 4. The three-dimensional nanofiber structures with high surface area and a large number of pores resulting from freeze-drying are responsible for the high porosity, which allows for applications of BC such as wound dressing material and controlled release of bioactive compounds [41]. The high porosity supports the good WHC and WAR of BC. These results are parallel to other researcher's findings [9,19,23,26–29,31,32,42,43].

3.3. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed to describe the thermal decomposition behavior of dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions. Three diverse stages of weight loss were associated with the TGA curve (Fig. 2). The first stage, up to 100 °C, there was a little weight loss almost about 5 % which is usually associated with the release of moisture or volatile compounds from BC structure and also the evaporation of adsorbed water of the surface of freeze-dried BC sample. From 100 °C to 200 °C, the BC mass was almost unchanged. On the second stage the major peak was observed around 350 °C due to cellulose thermall degradation processes, such as decomposition, depolymerization, and dehydration, of glycosyl units followed by the formation of burned rests and residues. The ultimate phase of weight reduction took place at temperatures exceeding 750 °C, revealing the oxidation and decomposition of the combusted remnants into lighter gaseous substances [19,44]. TG analysis confirmed that freeze-dried BC produced under the stirred fermentation method was very stable and had no degradation up to 200 °C. This high thermal stability of BC makes it a suitable additive in products that are subjected to relatively high thermal processes. It should be noted that the drying conditions of BC are effective on its thermal stability. Our results are consistent with the findings of other researchers who have studied the thermal properties of freeze-dried bacterial cellulose [19,30,31].

3.4. Fourier transforms infrared spectroscopy (FTIR)

Structural analysis of polymorphic freeze-dried BC produced by *Komagataeibacter xylinus* CH1 was carried out by FTIR analysis. Dried BC that was produced under stirred fermentation conditions, showed several typical slim, weak, broad, and strong bands for cellulose I in the different regions (Fig. 3(a)), the same as pure cellulose I ($C_6H_{10}O_5$) fingerprint. Considering that BC is a polysaccharide, the presence of many OH groups in the structure with hydrophilic properties is the main characteristic of its spectrum [28,32]. FTIR spectrum of BC produced by *Komagataeibacter xylinus* CH1 showed the peaks and stretching respectively as OH stretching (3358 cm^{-1}), CH stretching (2922 cm^{-1}), CH2 stretching (2847 cm^{-1}), OH angular bending (1650 cm^{-1}), HOH angular bending (1645 cm^{-1}), CH2 symmetric (1452 cm^{-1}), CH symmetric (1372 cm^{-1}& 1430 cm^{-1}), CH2 symmetric (1316 cm^{-1}), CO, COH stretching (1162 cm^{-1}), CO, COC stretching (1111 cm^{-1}, 1059 cm^{-1}, & 1034 cm^{-1}), CH symmetric (902 cm^{-1}), CH2 asymmetric (796 cm^{-1}), and OH symmetric (670 cm^{-1}, 617 cm^{-1}, & 559 cm^{-1}). Results confirmed that the BC produced by *Komagataeibacter xylinus* CH1, was pure and consistent with those of previous reports, which showed the typical bands of BC in FTIR spectra [19,28,30,32,37]. The purity of BC

Table 4
Physicochemical properties and crystallinity characteristics of freeze-dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions.

Sample	moisture content (%)	water absorption rate (%)	water-holding capacity (%)	Porosity (%)	water activity	bulk density ($g.ml^{-1}$)	tapped density ($g.ml^{-1}$)
Freeze-dried BC	93.022± 0.472	569.473± 3.739	1333.016± 3.680	166.247± 2.055	0.296±0.030	0.013±0.001	0.019±0.001

Peak position (°2θ) (1 0 1, 1 0¯1, 0 0 2)	d spacing (°A) (1 0 1, 1 0¯1, 0 0 2)	Crystallite size (°A) (1 0 1, 1 0¯1, 0 0 2)	Crystallinity Index (CI%)	Degree of crystallinity (XC%)
(14.70°, 16.95°, 22.70°) 2θ	(6.02°, 5.22°, 3.91°) °A	(89°, 89.2°, 90°) °A	63.81 %	73.42 %

Data are expressed as mean ± SD, n = 3.

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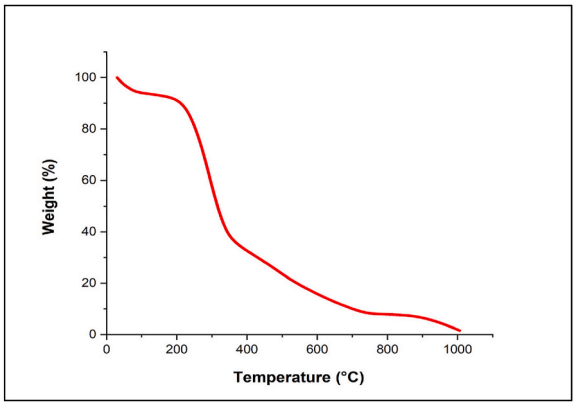

Fig. 2. TGA spectrum of freeze-dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions.

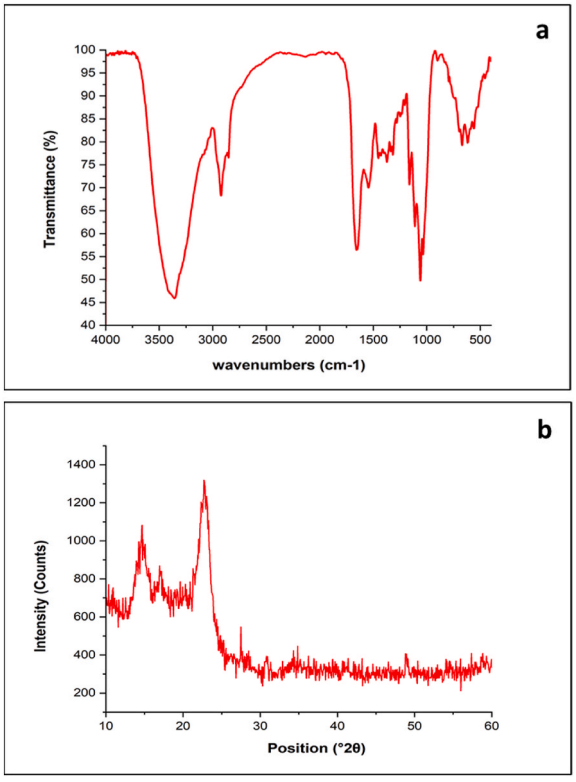

Fig. 3. (a) FTIR spectrum, (b) X-ray diffractogram of freeze-dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions.

was also confirmed by cellulase enzymatic hydrolysis process, that all the BC pellicles were hydrolyzed, and a clear liquid was appeared.

3.5. X-ray diffraction (XRD)

According to X-ray diffractogram, crystalline peaks and amorphous haloes can be seen in the structure of freeze-dried BC (Fig. 3 (b)). The obtained XRD pattern was very similar to the native cellulose I pattern, with peaks positions at 2θ of 14.4°, 16.7° and 22.6° [19]. The peaks positions were at 2θ of 14.70°, 16.95° and 22.70°. This diffractogram confirmed 3 main characteristic peaks standing for crystal planes 101, 10 $\bar{1}$, and 002, d-spacing 6.02°, 5.22°, and 3.91° A respectively which displayed the typical profile of the cellulose I [18,29] (Table 4). The crystallinity index has been used to define the relative amount of crystalline material in cellulose and degree of crystallinity is the amount of structurally ordered regions and areas in cellulose [45]. In our study the CI and XC values were

63.81 % and 73.42 % respectively. The XC value of BC reported by Ref. [45] was 41 %. The CI value of the BC under stirred method by *Komagataeibacter* sp. nov. CGMCC 17276, by Ref. [37] was 66.42 % and it was 81.2 % in the study by Ref. [41]. The decrease of the CI value of BC under stirred fermentation conditions may be the result of the shearing force [37].

3.5.1. Scanning electron microscopy (SEM)

Morphology analysis of BC was carried out by Scanning electron microscopy (SEM), under 100X, 250X, 1000X, 2500X, 5000X, and 10000× magnification. SEM images show fibrous with irregular size and shape of BC produced by stirred conditions. Under magnification of 2500× and above, a reticulated structure consisting of ultra-fine nanocellulose ribbon-like fibrils can be clearly distinguished (Fig. 4). The strands are entangled and curved resulting in a reticulated denser structure under stirred conditions. These fibrils form a porous structure. New chains of BC aggregate to form sub-fibrils, microfibrils, bundles, and ribbons [46]. The morphological changes in BC beads affect the microstructures and numerous properties like the crystallinity, water holding capacity, water absorption rate, porosity, and moisture content [32,41]. Similar observations reported on the bacterial cellulose produced by other bacterial strains under dynamic fermentation technique [8,32,47].

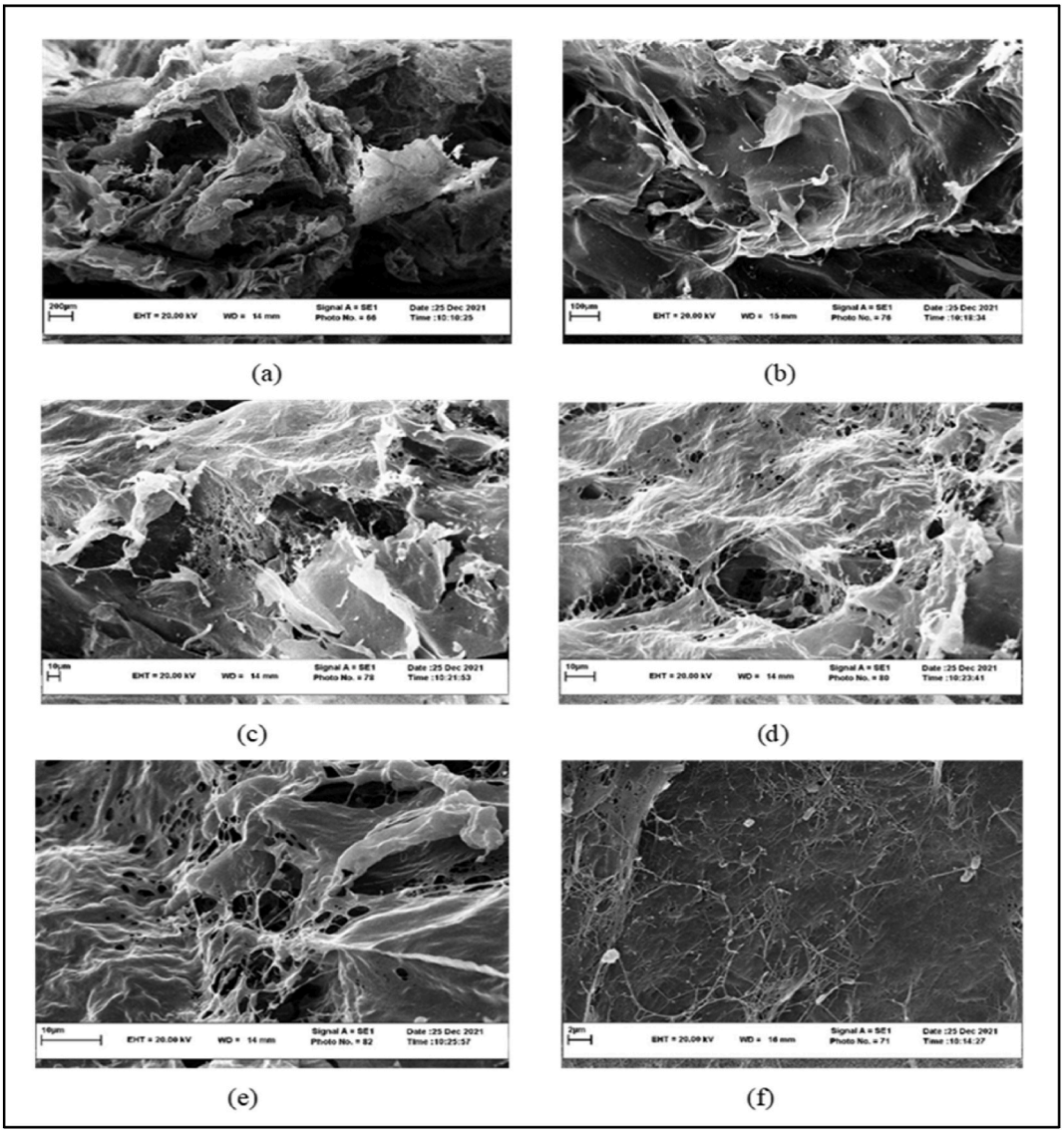

Fig. 4. SEM micrographs of freeze-dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions: (a) 100X, (b) 250X, (c) 1000X (d) 2500X, (e) 5000X and (f) 10000× magnification.

3.6. Color measurement

Regarding the color measurement, color of freeze-dried BC was reported as lightness (L* value) 84.839 ± 4.154, redness (value a*) 3.740 ± 1.552 and yellowish (b* value) 9.584 ± 2.200. The optical characteristics play a crucial role, especially when the powder is used in food products, as these factors significantly impact consumer satisfaction [48].

3.7. Dynamic rheological properties

The viscoelastic behavior of freeze-dried BC was assessed on 1 %, 1.5 %, and 2 % suspensions. The strain sweep measurements were achieved to determine the linear viscoelastic region where G′ and G″ were practically constant. Within the experimental range of frequency, all three samples showed an initial linear viscoelastic region (LVE) where G′ and G″ are independent of the strain and G'>G″, showing a solid-like behavior, while after the crossover point at 10 % strain, they showed an opposite behavior with G″ higher than G' (Fig. 5(a)). With increasing the gum concentration, the strain at which G′ and G″ are crossed over was increased. This shows that higher concentrations of gum increase the strength of the system. The G′, G″ and tan δ values for different concentrations of freeze-dried BC at LVE are reported in Table 5. Loss tan (tan δ) is an appropriate rheological parameter to characterize the viscoelastic behavior of the samples. tan δ less and higher than 1 shows a predominantly elastic and viscous behaviors, respectively [49]. Tan δ values of all the samples were less than 1 but higher than 0.1 indicating the presence of elastic structure in the weak biopolymer gel. The lowest amount of Loss tan was observed for 1 % concentration, indicating more elastic behavior than other concentrations.

The mechanical spectra of freeze-dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions as a function of frequency is shown in Fig. 5 (b). All the samples showed a typical weak gel-like behavior where the magnitude of G′ and G″ slightly has frequency dependency [50]. G′ and G″ were parallel through the studied range of frequency; while the elastic behavior dominated the viscous one at all frequencies. Viscous modulus showed more dependency on frequency comparing to elastic modulus.

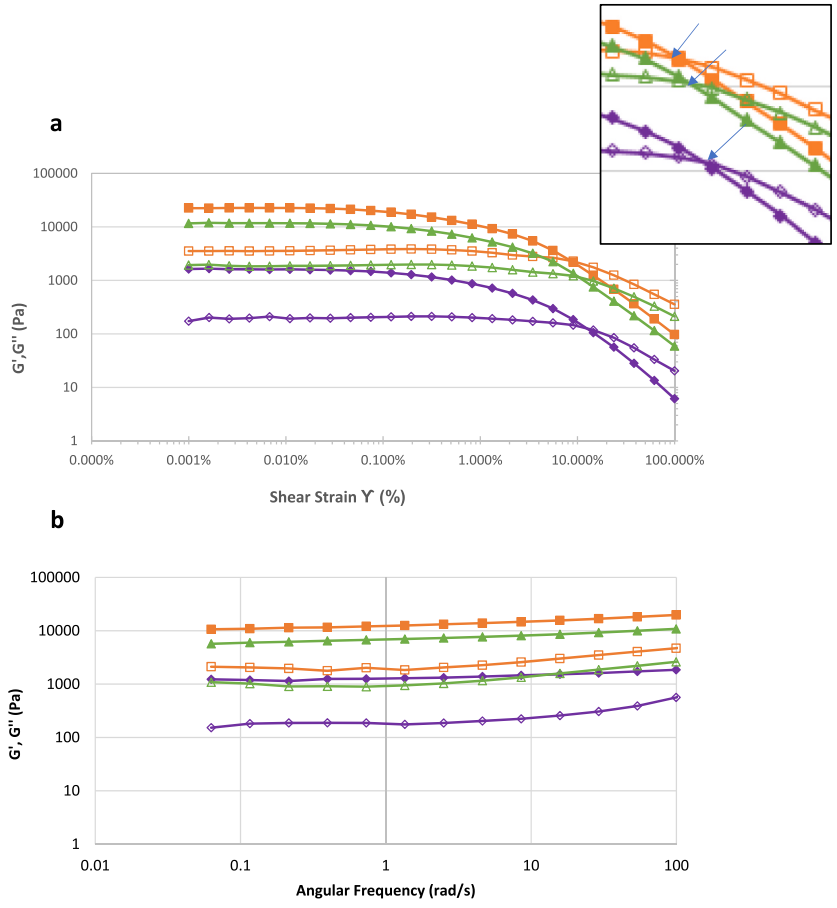

Fig. 5. (a) Strain sweep dependency of storage modulus (G′) and loss modulus (G″) at f = 1 H z, (b) frequency sweep of storage modulus (G′) and loss modulus (G″) at shear strain = 1 %.for 1 %, 1.5 % and 2 % freeze-dried BC suspensions produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions (G′(1 %); G'' (1 %); G' (1.5 %); G'' (1.5 %); G' (2 %); G'' (2 %)).

Table 5
Power law and viscoelastic parameters in frequency sweep (f = 6.28 rad/s) tests of freeze-dried BC produced by *Komagataeibacter xylinus* CH1 under stirred fermentation conditions.

Sample	G′ (Pa)	G″ (Pa)	tan δ	K (mPa)	n	R^2
Con 1 %	1420	215	0.151	11.50 ± 1.28	0.17 ± 0.02	0.99
Con 1.5 %	14160	2395	0.169	316.08 ± 17.67	0.07 ± 0.01	0.98
Con 2 %	7920	1200	0.151	316.10 ± 10.05	0.21 ± 0.01	0.9874

Loss tan is directly related to the energy lost per cycle divided by the energy stored per cycle [51]. Tan δ values reported in Table 5 were smaller than 1 (0.151–0.169) indicating that the elastic component dominates the viscous one in all three weak biopolymer samples. For all three concentrations, G′ was always higher than G″ and it was not dependent on concentration and as expected an increase in both G' and G" was detected when concentration is increased. The highest viscoelastic moduli value was observed for 1.5 % concentration, following by 2 % and 1 %, respectively. Previously [29], reported that the rheological properties of the BC pellicles were more affected by the network structure of the nanocellulose fibers than by fiber concentrations.

3.8. Steady flow behavior

The data of apparent viscosity vs. shear rate in the range of 0.1–100 s^{-1} were fitted by Power law model for 1 %, 1.5 %, and 2 % concentrations of BC suspensions. All percentages of bacterial cellulose suspensions showed a non-Newtonian pseudoplastic behavior; i.e., viscosity reduced with increasing shear rate (data not shown). Moreover, the viscosity value increased with increasing concentration in all samples (2 % > 1.5 % > 1 %) which was in agreement with the results reported by Ref. [52]. Power law model properly fitted the shear stress-shear rate and apparent viscosity-shear rate data (R^2 = 0.98–0.99). The rheological parameters of the Power law model are reported in (Table 5). Higher consistency coefficient was observed for higher levels of BC concentrations. The increased density of hydrogen bonding connections within the BC network [19].

4. Conclusion

The current investigation has resulted in an improved yield of freeze-driedbacterial cellulose (BC) production under stirred fermentation conditions, reaching 45.543 gL^{-1}. This was achieved by using a 3.013 % VV^{-1} inoculation of *Komagataeibacter xylinus* CH1 strain (isolated from kombucha SCOBY), 0.516 % of NIW as an alternative carbon source, and a 7-day fermentation period. NIW, a cost-effective and inexpensive carbon source, can be utilized by bacteria for the synthesis of high-value BC, making it a smart and economical approach. While BC production under static conditions is a common technique, the results of this research demonstrate that the stirred fermentation method exhibited excellent physicochemical and rheological characteristics, with high efficiency suitable for commercial applications. Undoubtedly, further research in this field will be beneficial in increasing access to this valuable biological composition and ensuring sustainable food security through the development of waste consumption management.

Data availability statement

The datasets generated and analyzed during the current study is available in the [NCBI] repository at, https://www.ncbi.nlm.nih.gov/nuccore/?term=PP716809:PP716815[accn]. https://www.ncbi.nlm.nih.gov/nuccore/PP716813.1.

CRediT authorship contribution statement

Azadeh Khiabani: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mahboobe Sarabi-Jamab:** Writing – review & editing, Validation, Supervision, Project administration, Investigation. **Monir-sadat Shakeri:** Writing – review & editing, Validation, Investigation. **Abolfazl Pahlevanlo:** Writing – review & editing, Validation, Investigation. **Bahareh Emadzadeh:** Writing – review & editing, Validation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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