



A comparison of kombucha SCOBY bacterial cellulose purification methods

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Abstract

Kombucha SCOBY pellicle formed as a waste product in the kombucha tea fermentation industry can be used as a potential source for bacterial cellulose. We have compared four simple, scalable purification methods for the purification of this jelly like cellulosic matrix containing bacteria, yeast cells, proteins and polyphenols as impurities. The method using two successive 1.0 M NaOH washings at 90 °C, followed by the treatment with 1.5% (w/w) aq. NaOCl for 2 h, was the most effective method and gave the purified cellulose sample with the highest leucometer whiteness value of 81.4 ± 4.8 . The purified cellulose samples were analyzed using FT-IR, TGA, SEM, EDX, X-ray and water absorption at room temperature.

Keywords Kombucha · Scoby · Bacterial cellulose · Hydrogen peroxide · Sodium hypochlorite

1 Introduction

Kombucha is the common name given to a fermented lightly alcoholic beverage prepared using sweetened black or green tea. Sometimes the beverage is called kombucha tea as well to differentiate from the bacteria and yeast culture used in the fermentation process. This centuries old beverage is believed to have been originated in north-eastern china. The current broad attention to this classical beverage due to renewed interest in supposed health benefits has resulted a number commercial scale productions and a home brewing renaissance [1, 2]. Kombucha culture is composed of a symbiotic growth of acid producing bacteria and osmophilic yeast species in a zoogloeal mat. During the kombucha tea fermentation a gelatinous, cellulose-based biofilm or a pellicle is formed at the air–liquid interface. This biofilm is a Symbiotic Culture of Bacteria and Yeast (SCOBY) and commonly known as a tea mushroom as well. The microbial populations in a SCOBY may

vary, but generally include *Acetobacter* bacterial species, various *Saccharomyces* and a number other types of yeasts [3]. Within this culture, anaerobic ethanol fermentation by yeast, organic acid fermentation and aerobic ethanol oxidation to acetate through bacteria can all take place concurrently along an oxygen gradient. In addition, *Acetobacter* may well polymerize glucose residues forming a bacterial cellulose mat supporting the microbial culture [3, 4].

In recent years, with the surge of the kombucha beverage industry, a number of research groups around the globe have studied the microbial culture, composition of the fermented liquid as well as the bacterial biofilm at the water–air interface. The major components of the fermented media are gluconic, acetic, lactic acids and ethanol; in addition, minor amounts of proteins, tea polyphenols, minerals and vitamins (B1, 2, 6, 12 and C) are also reported [4, 5]. The pellicle formed at the air–liquid interface is mainly cellulose. Similar bacterial cellulose forms

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are known for several decades and is traditionally isolated from nata de coco, a gelatinous southeast Asian dessert made from coconut water [6]. In this preparation coconut water serves as the carbon source for bacteria *Acetobacter xylinum* and later converted to extracellular cellulose [6]. In comparison with plant cellulose, microfibrils of bacterial cellulose are generally about 100 times smaller than in plant cellulose and this fine structure leads to a number of appealing properties of bacterial cellulose, such as high water absorption capacity and strength [7, 8]. In addition, the well arranged three dimensional nano-fiber network in bacterial celluloses results the formation of hydrogel sheets with high surface area, abundant surface hydroxyl groups, high porosity and good chemical-modifying capacity [9, 10]. As a result bacterial cellulose has found in numerous applications such as wound dressing [11, 12], raw material for food and as food packaging [13], bioreactor, bio-processing [14], optoelectronics [15], polymer electrolyte membranes [16], and many medical applications such as: implants and scaffolds for tissue engineering of cartilage as well as carriers for drug delivery [17–19].

Our interest in processing cellulosic biomass for renewable fuels and chemical feedstock applications has led us to study the kombucha pellicle formed as a waste product in the growing kombucha beverage industry as a potential new sources of bacterial cellulose [20–22]. Under this program, we have studied the probable industrially scalable methods for the purification of kombucha pellicle to produce bacterial cellulose. In this publication, we present a comparison of four simple methods and identification of the best conditions for the purification of bacterial cellulose formed as a waste product in the kombucha tea industry.

2 Experimental

2.1 Materials and instrumentation

The starting kombucha SCOBY was purchased from www.poseymom.com. Lipton black tea (100 tea bags, total 226 g) was purchased from local grocery store. Sodium hydroxide (> 99%), sucrose (> 99%), sodium hypochlorite solution (6%) and hydrogen peroxide (3%) were purchased from Aldrich Chemical Co. Attenuated total reflection infrared (ATR-IR) spectra of cellulose films were recorded in the 650–4000 cm^{-1} range on a Smiths IdentifyIR spectrometer with a diamond ATR (Danbury, CT, USA). Thermogravimetric analysis was carried out in air using a Perkin Elmer Diamond TG/DTA, High Temp 115. The mass of the thin film piece used for a TG scan was approximately 10 mg, and platinum crucibles were used in all experiments. The TG curves were recorded in the 25–600 °C temperature

range using a scanning rate of 20 °C min^{-1} . Cellulose film thickness was measured using a Pittsburgh instruments digital micrometer model 895. Whiteness of purified cellulose films were measured using Landtek GM-6 Leucometer. Scanning electron microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX) analyses were performed using a JEOL JSM-6010LA using InTouchScope software. The backscattered electron images was collected using an accelerating voltage of 10 kV and a load current $\sim 90 \mu\text{A}$ with a working distance of 9 mm. EDS spectra were collected at a magnification of 500 \times and the analyzed area was 0.15 mm^2 (110 $\mu\text{m} \times 135 \mu\text{m}$). Qualitative and quantification elemental composition was performed with the characteristic x-ray using a silicon-drift detector. The semi-quantification is based on the theoretical correction of the ZAF effect. X-ray diffraction experiments were performed using a Bruker D8 Davinci spectrometer, wavelength 1.54060 Å, with 40 kV, 15 mA/Cu source, and scan type: 2 θ .

2.2 Preparation of the kombucha pellicle

Tea was brewed by using Lipton black tea (11.3 g, five tea bags) and 1.0 L of boiling water and allowed to stand for 20 min. The tea bags were then removed, sucrose (100 g, 0.292 mol) was dissolved in tea and allowed to cool to 37 °C and kombucha SCOBY was added. The sweetened tea with the starting SCOBY was then covered with a cotton cloth and allowed to stand in a dark cupboard for 10 days. Then the new kombucha pellicle formed at the air–water interface was carefully removed, washed with deionized water (2 \times 500 mL) and pat dried with Kleenex tissues and used for the purification experiments.

2.3 Purification of the bacterial cellulose in kombucha pellicle

The kombucha pellicle was cut into four approximately equal in size quarters and labeled as A, B, C and D and purified using four different methods.

2.3.1 Purification of kombucha pellicle portion A

Kombucha pellicle portion A was placed in 50 mL of 1.0 M aqueous sodium hydroxide solution and allowed to stand at room temperature (23 °C) for 1.0 h. Then removed from the sodium hydroxide solution and washed with deionized water (6 \times 500 mL), pat dried with Kleenex tissues and weighed. The pellicle portion was then placed in a second 50 mL of 1.0 M aqueous sodium hydroxide bath, allowed to stand at room temperature (23 °C) for 1.0 h washed with deionized water dried and weighed as in the earlier treatment. Next the pellicle portion A was placed in 50 mL of 1.5% (w/w) aqueous hydrogen peroxide, allowed to stand

at room temperature (23 °C) for 2.0 h. Then removed from the hydrogen peroxide bath and washed with deionized water (6 × 500 mL), pat dried with Kleenex tissues and weighed. Finally, the pellicle portion A was transferred to a watch glass and dried in an oven at 50 °C for 20 h and weighed. The weights after each treatment are shown in Table 1.

2.3.2 Purification of kombucha pellicle portion B

Kombucha pellicle portion B was placed in 50 mL of 1.0 M aqueous sodium hydroxide solution and allowed to stand at room temperature (23 °C) for 1.0 h. Then removed from the sodium hydroxide solution and washed with deionized water (6 × 500 mL), pat dried with Kleenex tissues and weighed. The pellicle portion was then placed in a second 50 mL of 1.0 M aqueous sodium hydroxide bath, allowed to stand at room temperature (23 °C) for 1.0 h washed with deionized water dried and weighed as in the earlier treatment. Next the pellicle portion B was placed in 50 mL of 1.5% (w/w) aqueous sodium hypochlorite, allowed to stand at room temperature (23 °C) for 2.0 h. Then removed from the sodium hypochlorite bath and washed with deionized water (6 × 500 mL), pat dried with Kleenex tissues and weighed. Finally, the pellicle portion B was transferred to a watch glass and dried in an oven at 50 °C for 20 h and weighed. The weights after each treatment are shown in Table 1.

2.3.3 Purification of kombucha pellicle portion C

Kombucha pellicle portion C was placed in 50 mL of 1.0 M aqueous sodium hydroxide solution thermostated at 90 °C for 1.0 h. Then removed from the sodium hydroxide solution and washed with deionized water (6 × 500 mL), pat dried with Kleenex tissues and weighed. The pellicle portion was then placed in a second 50 mL of 1.0 M aqueous sodium hydroxide bath thermostated at 90 °C, allowed to stand for 1.0 h, washed with deionized water, dried and weighed as in the earlier treatment. Next the pellicle

portion C was placed in 50 mL of 1.5% (w/w) aqueous hydrogen peroxide, allowed to stand at room temperature (23 °C) for 2.0 h. Then removed from the hydrogen peroxide bath and washed with deionized water (6 × 500 mL), pat dried with Kleenex tissues and weighed. Finally, the pellicle portion C was transferred to a watch glass and dried in an oven at 50 °C for 20 h and weighed. The weights after each treatment is shown in Table 1.

2.3.4 Purification of kombucha pellicle portion D

Kombucha pellicle portion D was placed in 50 mL of 1.0 M aqueous sodium hydroxide solution thermostated at 90 °C for 1.0 h. Then removed from the sodium hydroxide solution and washed with deionized water (6 × 500 mL), pat dried with Kleenex tissues and weighed. The pellicle portion was then placed in a second 50 mL of 1.0 M aqueous sodium hydroxide bath thermostated at 90 °C, allowed to stand for 1.0 h washed with deionized water, dried and weighed as in the earlier treatment. Next the pellicle portion D was placed in 50 mL of 1.5% (w/w) aqueous sodium hypochlorite, allowed to stand at room temperature (23 °C) for 2.0 h. Then removed from the sodium hypochlorite bath and washed with deionized water (6 × 500 mL), pat dried with Kleenex tissues and weighed. Finally, the pellicle portion D was transferred to a watch glass and dried in an oven at 50 °C for 20 h and weighed. The weights after each treatment are shown in Table 1.

2.4 Analysis of purified kombucha pellicle portions A, B, C and D

The dried kombucha pellicle portions A, B, C and D were analyzed by FT-IR, TGA and SEM. The thickness of films were measured using a digital micrometer and the whiteness was measured using a Landtek GM-6 Leucometer. The water absorption of purified, dried kombucha pellicle portions A,B,C and D were measured by immersing a weighed portion of dried kombucha film (~ 100 mg) in deionized water and weighing the water absorbed sample

Table 1 Weights of kombucha pellicle portions A, B, C and D at various stages of purification

Sample	Initial weight (g)	After, 1.0 M NaOH, 23 °C, 1.0 h (g)	After, 1.0 M NaOH, 90 °C, 1.0 h (g)	After, 1.0 M NaOH, 23 °C, 2.0 h (g)	After, 1.0 M NaOH, 90 °C, 2.0 h (g)	After, 1.5% (w/w) H ₂ O ₂ , 23 °C, 2.0 h (g)	After, 1.5% (w/w) NaOCl, 23 °C, 2.0 h (g)	Dry weight (g)
A	40.11	43.86	–	46.38	–	46.65	–	1.10
B	41.77	47.15	–	48.77	–	–	48.19	1.52
C	39.62	–	42.93	–	44.89	44.76	–	1.06
D	44.37	–	47.48	–	50.32	–	47.67	1.54

at different time intervals after pat drying the film portion with Kleenex tissues.

3 Results and discussion

3.1 Purification of kombucha pellicle portions A, B, C and D

Kombucha pellicle portions A, B, C and D were purified using four different methods. The samples A and B were washed with 1.0 M aq. sodium hydroxide at room temperature, whereas, C and D were washed with the same sodium hydroxide at 90 °C for a similar time. Then A and C were subjected to hydrogen peroxide treatments and B and D were subjected to sodium hypochlorite treatments. The weights of pellicle portions A, B, C and D at various stages of purification are shown in Table 1. Aqueous sodium hydroxide treatment or alkaline treatment is the most widely used method for the purification of cellulose and hemicellulose from plant materials [23]. In this case aqueous sodium hydroxide is known to remove lignin by hydrolyzing ester bonds present between lignin and hemicellulose [23, 24]. Similarly, in the present experiments NaOH treatment may help in removing tannins and polyphenols as well as bacteria, yeast cells and proteins, that may be present in minor amounts in the kombucha pellicle. The NaOCl [25] and H₂O₂ [26] are well known bleaching agents in cellulose purification, therefore we have tested these reagents in bleaching the colored impurities in bacterial cellulose from Kombucha.

All samples showed gains in wet weights during sodium hydroxide washings, and this may be due to opening of pores in cellulose structure due to sodium hydroxide washings; allowing more water absorptions, as well as surface absorption of sodium ions. The dry weights of cellulose samples after purification are shown in the last column of Table 1. The hydrogen peroxide treated samples A and C showed much lower dry weights in comparison to the sodium hypochlorite treated samples B and D; this may possibly due to the oxidation of some cellulose with hydrogen peroxide and degradation of the polymer [26].

3.2 Analysis of purified kombucha cellulose samples A, B, C and D

The leucometer whiteness readings, film thickness, and Total Crystallinity Index (TCI) of purified kombucha cellulose samples A, B, C and D are in Table 2. The sample D purified using sodium hydroxide solution at 90 °C and sodium hypochlorite treatment showed the highest whiteness of 81.4 ± 4.8 . Sample A purified using sodium hydroxide solution at room temperature and hydrogen peroxide

Table 2 Leucometer whiteness readings, film thickness, and Total Crystallinity Index (TCI) of purified kombucha cellulose samples A, B, C and D

Sample	Leucometer whiteness reading ^a	Thickness ^a (mm)	Total Crystallinity Index (TCI) ^b
A	23.8 ± 1.6	0.42 ± 0.06	3.852
B	36.3 ± 1.5	0.41 ± 0.04	2.900
C	36.4 ± 2.3	0.33 ± 0.08	2.931
D	81.4 ± 4.8	0.27 ± 0.02	2.750

^aAverage of five readings in each determination

^bTotal Crystallinity Index (TCI) was measured using FT-IR spectroscopy, and the formula: $TCI = (\text{absorption at } 1373 \text{ cm}^{-1}) / (\text{absorption at } 2900 \text{ cm}^{-1})$ [27]. FT-IR spectra of samples are in the supplementary material

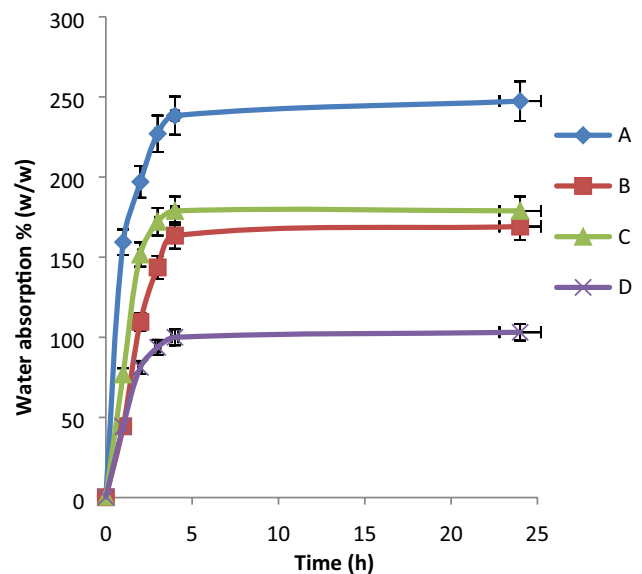


Fig. 1 The percent water absorption (w/w) of purified kombucha cellulose samples A, B, C and D at room temperature (23 °C)

treatment resulted in the lowest whiteness of 23.8 ± 1.6 and the darkest sample. In addition, the samples washed with sodium hydroxide at 90 °C (C and D) appears to be much thinner than samples washed with sodium hydroxide at room temperature. The total crystalline index (TCI) of all purified samples were calculated using FT-IR spectra as described by Kruer-Zerhus et al. [27] in their work on similar bacterial cellulose samples. The FT-IR spectra are included in the supplementary material and TCI values were calculated using the formula: $TCI = (\text{absorption at } 1373 \text{ cm}^{-1}) / (\text{absorption at } 2900 \text{ cm}^{-1})$ [27]. The whitest sample D showed the lowest crystallinity index of 2.750 whereas the darkest sample A showed the highest crystallinity index of 3.852. This is most likely indicating that

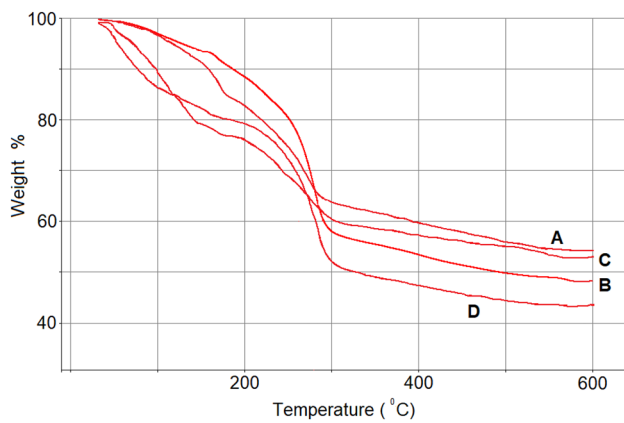


Fig. 2 Thermogravimetric analysis (TGA) curves of purified kombucha cellulose samples A, B, C and D

strong conditions, 1.0 M aq. sodium hydroxide at 90 °C and 1.5% sodium hypochlorite treatment used in the purification of sample D can cause reduction in crystallinity of the cellulose as well.

The percent water absorption (w/w) of purified kombucha cellulose samples A, B, C and D at room temperature

are shown in Fig. 1. All purified cellulose samples reached their maximum water absorption capacities after about 4 h. The highest water absorption of 247% was seen for sample A and the lowest percent of 103% was seen for sample D. This is the reversed trend of whiteness in the sample; in other words, the highly purified sample showed the lowest water absorption capacity. This may be explained as an increase in water absorption in cellulose as a result of impurities present in the cellulose sample. Furthermore, it is interesting to note that the samples B and C showing similar whiteness readings appears to have similar water absorption capacities.

The thermogravimetric analysis (TGA) curves of purified kombucha cellulose samples A, B, C and D are shown in Fig. 2. All samples showed a rapid decomposition and major weight losses around 270–280 °C range. The most purified (highest whiteness) sample D showed the lowest residue left at 600 °C of about 43%, whereas the least purified (lowest whiteness) sample D showed the highest residue of about 55% at 600 °C. This again indicates the inadequate purification in A, in which the impurities may have contributed to the formation of higher amount of un-burnt chars at 600 °C.

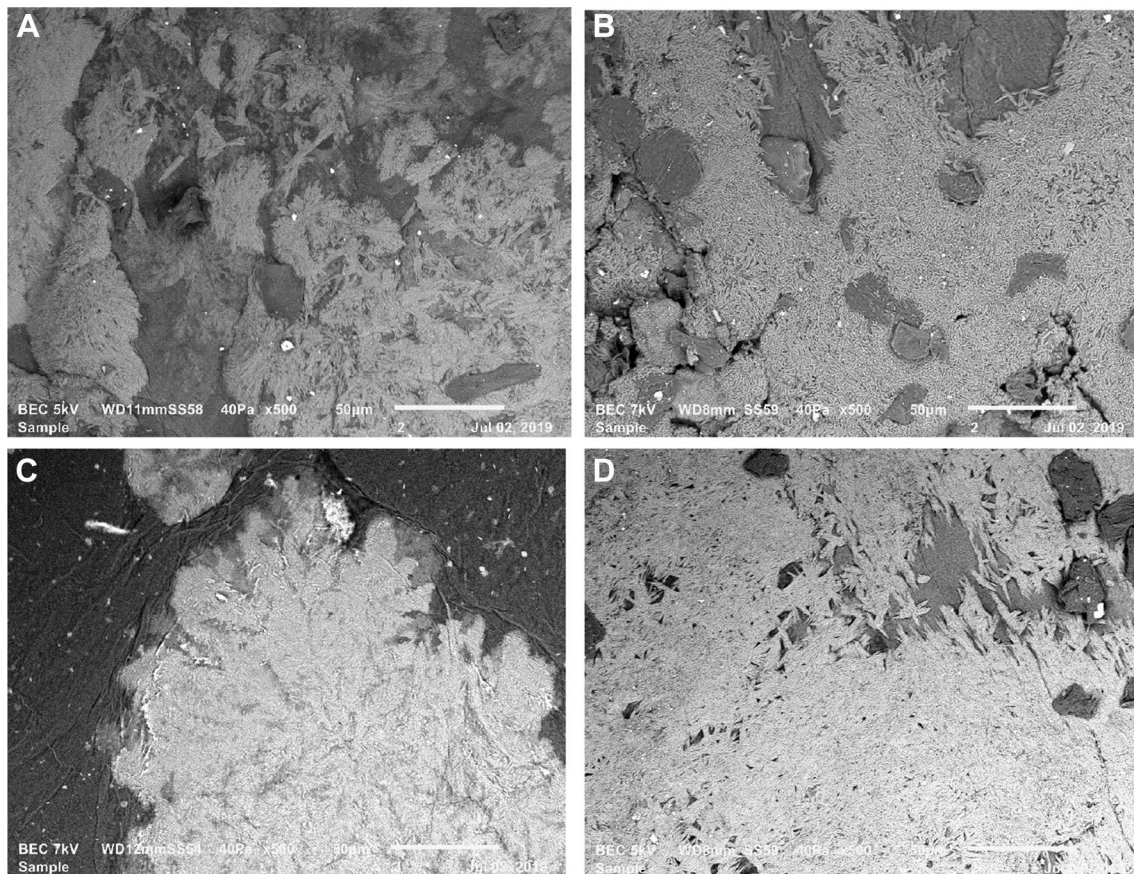


Fig. 3 SEM images of purified kombucha cellulose samples A, B, C and D

The SEM images of purified kombucha cellulose samples A, B, C and D are shown in Fig. 3. Surface C, O, Na and Cl elemental analysis % (w/w) composition data of purified kombucha cellulose samples A, B, C and D from Energy Dispersive X-ray Spectroscopy (EDX) is shown in Table 3. EDX was used to analyze surface elemental composition of purified cellulose samples and compare the changes in surface compositions due to different purification methods. The sample with the highest purity D showed wider homogeneous regions of cellulose fibrils and least amount of dark patches due to impurities. The surface analysis in Table 3 showed the presence of significant amounts of sodium on the surface of all purified cellulose samples, accumulated on the surface during the sodium hydroxide washings. The two samples B and D treated with sodium hypochlorite showed trace amounts of chlorine on the surface as well. The X-ray diffraction profile of kombucha cellulose sample D is shown in Fig. 4. The major diffraction peaks at 2θ values of 14.5° , 16.6° and 22.6° in the purified Kombucha cellulose are most likely associated with 110, 110 and 200 planes respectively as similar values are

reported for bacterial cellulose samples from *Acetobacter xylinum* [28, 29].

4 Conclusion

We have compared four simple and scalable methods for the purification of cellulosic matrix formed as a byproduct in the kombucha tea fermentation process. The purification methods tested involved repeated washings with 1.0 M aq. sodium hydroxide and bleaching using hydrogen peroxide or sodium hypochlorite. The method using two initial 1.0 M sodium hydroxide washings at 90°C , and a bleaching with 1.5% sodium hypochlorite for 2 h, was identified as the most effective technique, which produced the purified cellulose sample with the highest leucometer whiteness value of 81.4 ± 4.8 . In summary, we have shown that sodium hydroxide washings and dilute sodium hypochlorite treatment can be applied for the purification of bacterial cellulose from kombucha SCOBY pellicle formed as a waste product in kombucha tea fermentation industry.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Table 3 Surface C, O, Na and Cl elemental analysis % (w/w) composition data of purified kombucha cellulose samples A, B, C and D from Energy Dispersive X-ray Spectroscopy (EDS) (H atoms are not observed in EDS)

Sample	C	O	Na	Cl
A	28.08	49.01	22.91	–
B	27.25	48.53	23.29	0.93
C	33.26	48.47	18.27	–
D	28.28	53.24	28.00	0.48

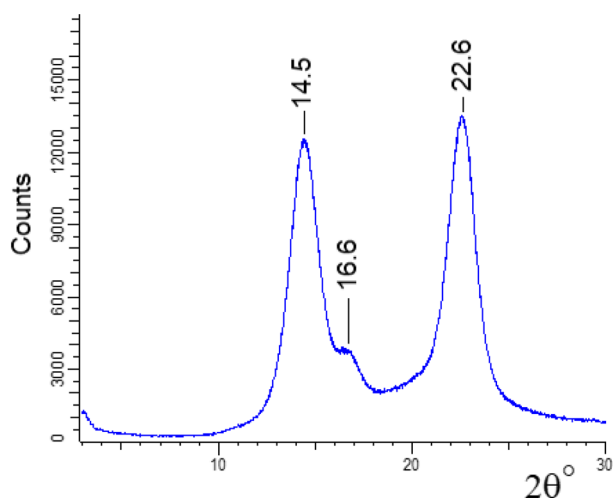


Fig. 4 X-ray diffraction profile of kombucha cellulose sample D

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