International Food Research Journal 19(1): 153-158 (2012)

Microstructure and physical properties of microbial cellulose produced during fermentation of black tea broth (Kombucha). II.

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Abstract: In this study, we evaluated and characterized microbial cellulose produced from Kombucha after eighth day of fermentation by employing SEM, FTIR, X-ray diffractometry, adsorption isotherm, and by measuring the swelling properties. Results on SEM revealed microbial cellulose layer to be composed of a compact cellulose ultrafine network like structure. FTIR spectra showed the presence of a characteristic region of anomeric carbons (960 – 730 cm^{-1}), wherein a band at 891.59 cm^{-1} confirmed the presence of β, 1-4 linkages. Results of FTIR spectra also showed microbial cellulose to be free from contaminants such as lignin or hemicellulose, which are often present in plant cellulose. X-ray diffraction studies exhibited the overall degree of crystallinity index for MCC to be slightly lower than that of microbial cellulose. Results on swelling properties indicated microbial cellulose to possess higher fiber liquid retention values (10-160%) compared to commercial MCC (5-70%). The adsorption isotherm curves showed similarities between microbial cellulose with that of pure crystalline substance. Overall, results obtained in this study were comparable with the commercial microcrystalline cellulose, indicating that the process developed by us can be explored industrially on a pilot scale.

Keywords: Microbial cellulose, SEM, FTIR, X-ray diffractometry, adsorption isotherm, swelling properties

Introduction

Kombucha is one of the highly popular fermented traditional beverages consumed for potential health benefits. Consumption of Kombucha is reported to cure some of the common ailments like arthritis, indigestion, various types of cancer, hepatotoxicity, etc. (Sreeramulu *et al*., 2000; Pauline *et al*., 2001; Hiremath *et al*., 2002). Kombucha fermentation is brought about by the symbiosis of yeast species and acetic acid bacteria (Teoh *et al*., 2004) and the harboring microbes or the starter culture has been declared to be safe by the US Food and Drug Administration (CDC, 1996), which further paves the way for exploring from consumer's safety prospects.

Cellulose is one of the most abundant naturally occurring polysaccharide which forms an integral part of most plant materials. However, in several instances, commercially available plant based cellulose are impure due to the presence of high amounts of lignin or hemi-cellulose. Compared to the plant cellulose (PC), bacterial cellulose (BC) can be synthesized (extracellularly) by bacteria such as *Acetobactor xylinum* and *Sarcinia. BC* is reported to possess several advantages which include: high purity, better mechanical strength, crystallinity and hydrophilicity with same chemical structure as that of PC (Yoshinaga *et al*., 1997; Phisalaphong and Jatupaiboon, 2008). Considering these facts, it is highly essential and a pre-requisite to characterize the microbial cellulose produced from Kombucha for commercial use and applications. In the present study, investigations were carried out to characterize the microbial cellulose that was produced during production of Kombucha. To the best of our knowledge, this is the first time that this type of characterization of microbial cellulose has been reported from Kombucha.

Materials and Methods

Materials

Black tea (Boh, Superior Cameron Highlands Tea, Malaysia) was used as the substrate for the fermentation and starter culture was obtained from a local commercial source. Sucrose, used as the carbon source was of food-grade (Gula Prai, Malayan Sugar Mfg. Co. Bhd., Malaysia). Commercial microcrystalline cellulose (MCC, Avicel, Philadelphia, USA) was used as comparison in cellulose characteristic studies.

Maintenance of tea fungus sample, and preparation of tea broth for fermentation

Maintenance of tea fungus samples and preparation of tea broth for fermentation was carried out as per the procedures detailed earlier in the first part of this paper series (Goh *et al*., 2011).

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Scanning electron microscopy (SEM) studies

The cellulosic pellicle formed on the eighth day of fermentation was cut into thin slices and washed with double distilled water. The washed cellulose fragments were centrifuged at 4500 rpm for 2 min. (Kubota 5100, Tokyo, Japan) and the supernatant was discarded. Centrifugation was repeated 4 times to get rid of the adhering mucus. Sample was then freeze-dried before analysis using Scanning Electron Microscope (Model S-360, Leica, Cambridge, U.K.) to study its cellulose network, and distribution of acetic acid bacteria and yeasts. SEM studies were also performed with the commercial MCC to compare with the microbial cellulose produced from Kombucha.

Fourier transformed infrared spectroscopy (FTIR)

The cellulose pellicles, harvested on the eighth day of fermentation were rinsed with distilled water (3 times), cut into slices and were freeze-dried. The freeze-dried sample was then ground into powder form for crystallinity analysis.

The analysis of crystallinity for the microbial cellulose was carried out by FTIR spectrometer (Nicolet Avator 360-FT-IR Thermo Electron Inc., San Jose, CA, USA) employing the method of Abbott *et al*. (1988). For data acquisition, software OMNIC 5.1 was used. A total of 200 scans were taken with a resolution of 4 cm^{-1} in order to produce the spectra. The commercial MCC powder was also scanned for comparison purpose.

X-ray diffractometry

The freeze-dried samples were ground into powder for x-ray diffractometry analysis. The diffractograms were recorded at room temperature (Siemens-5000 diffractometer, Siemens 5000, Karlsruhe, Germany) using Ni-filtered Cu K_α radiation ($\lambda = 1.54$ A°). The operating voltage and current were 40 kV and 18 mA, respectively. Data were collected at 0.02° 2θ intervals. The commercial MCC was also sent for x-ray diffraction for the purpose of comparison.

Degree of crystallinity was calculated from the diffracted intensity data using empirical method proposed by Segal *et al*. (1959):

$$(Cr.I.) = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

where, Cr.I. is the degree of crystallinity, I_{002} is the maximum intensity of the (002) lattice diffraction and I_{am} is the intensity diffraction at 18-2θ degrees.

Swelling properties

The liquid retention value (LRV) was determined analogous to water retention value (Tappi Useful Methods, UM 256, 54/1991). A known amount (1 g) of sample was mixed with 40 g of different solutions (water, acetone, dimethyl sulfoxide, and acetic acid). The suspension was allowed to stand for 2 hours, and then centrifuged at 3500 rpm for 30 minutes (Kubota-5100, Tokyo, Japan). After centrifugation, the supernatant was drained and the wet sample precipitate was weighed. The results were expressed as gram of water per gram of sample. The LRV experiment for both microbial cellulose and the commercial MCC was run in duplicate.

Adsorption isotherms

Sorption isotherms of microbial cellulose and the commercial MCC were determined at 30°C according to the procedure described by Spiess and Wolf (1983), with minor modifications. Both microbial cellulose and MCC samples were pre-dried in vacuum desiccators over P_2O_5 at room temperature (30 ± 2°C) for 7 days to obtain "zero water content. The dried samples (200 mg each) were weighed, to the nearest 0.0001 g, into pre-weighed weighing bottles. The dried samples, in quadruplicates, were equilibrated in air-tight 1-litre Kilner jars containing different saturated salt solutions of known relative vapour pressure (RVP) at 30 °C. The saturated salt solutions used were lithium chloride, potassium acetate, magnesium chloride, potassium carbonate, sodium bromide, strontium chloride, sodium chloride, and potassium chloride, with RVP of 0.11, 0.22, 0.32, 0.43, 0.56, 0.69, 0.75 and 0.84, respectively. Samples were weighed daily. "Equilibrium was assumed to have achieved when the change in weight did not exceed 0.1% for 3 consecutive weighing. Moisture content (dry basis) was calculated from the weight gained at this point.

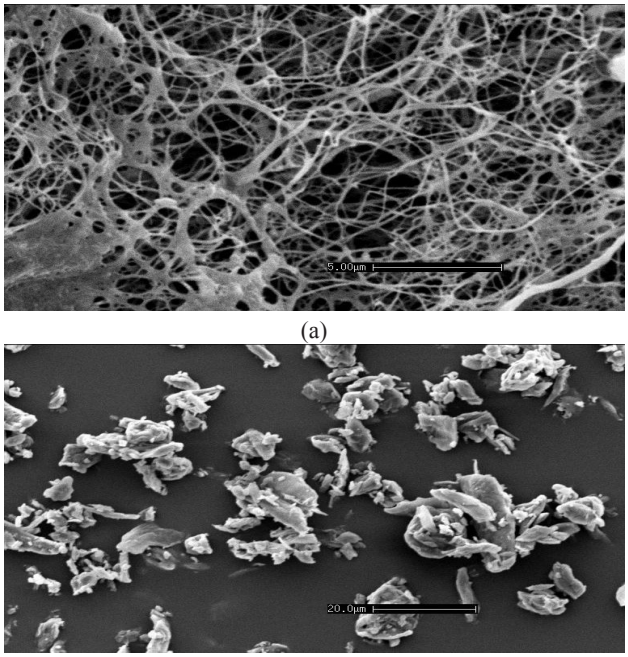
Results and Discussion

Characterization of the microbial cellulose was conducted and compared with that of the commercial MCC. The MCC was chosen as the reference in this study because MCC is purified native cellulose and it is not a chemical derivative, and there has been no chemical modification of the cellulose molecule (Thomas and Pourcelot, 1993).

Scanning electron microscopy (SEM)

Figure 1a shows the scanning electron micrograph of microbial cellulose, which is characterized by an ultrafine network structure and the microbial cellulose layer, constituted by a compact cellulose network structure which is on par with the observation of

Klemm *et al*. (2001). According to Iguchi *et al*. (2000), the cellulosic pellicle comprised of random assembly of microfibrils of less than 100 A° in diameter such as seen in the electron micrograph of freeze-dried Kombucha's cellulosic pellicle.

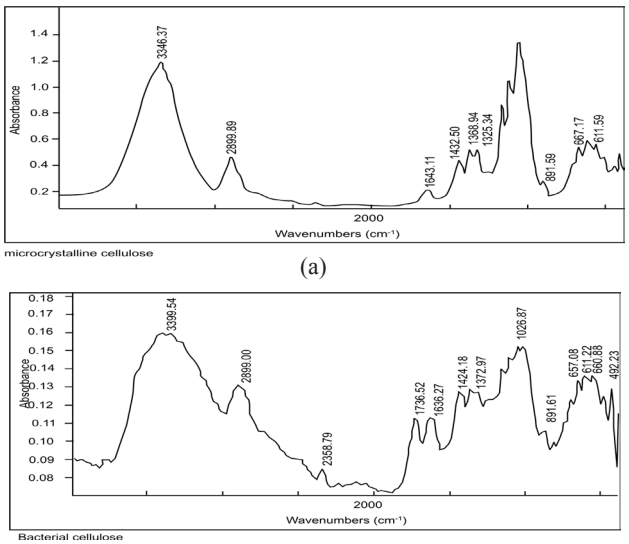


(a)

(b)

Figure 1. (a) Scanning electron micrograph of bacterial cellulose network. (Magnification: 5,000×); (b) Scanning electron micrograph of the commercial MCC. (Magnification: 1,000×)

Figure 1b shows the scanning electron micrographs of the commercial MCC. Compared to the produced microbial cellulose, MCC pose a very different structure. The primary particles forming the MCC aggregates were bigger in size (Figure 2) and therefore showed a low degree of coalescence between boundaries (Kothari *et al*., 2002). In addition, the MCC aggregates showed less smooth surfaces and were also less densely packed.



(a)

Figure 2. (a) FTIR spectra of the commercial MCC; (b) FTIR spectra of the bacterial cellulose.

Fourier Transformed Infrared Spectroscopy (FTIR)

The position and intensity of absorption bands of a substance are extremely specific to that substance. Like a fingerprint of a person, the infrared spectrum by FTIR is highly characteristic for a substance (Gunzler and Gremlich, 2002) and can be used to identify the microbial cellulose by comparing with the reference commercial MCC.

In this study, the chemical nature of the microbial cellulose polymer was confirmed by the infrared spectra of the MCC. Figure 2a shows the FTIR spectra of the commercial MCC and Figure 2b shows the FTIR spectra of the microbial cellulose. In general, both the spectra of MCC and microbial cellulose depict a similar trend. Absorbance spectra for microbial cellulose and MCC in the region 4000-450 cm^{-1} are reported in Figures 2a and 2b. The absorbance bands are consistent with that of a glucose polymer and their assignments were carried out based on data from literature (Bertocchi *et al*., 1997).

The characteristic region of anomeric carbons (960 – 730 cm^{-1}) was identified, where a band at 891.59 cm^{-1} confirms the presence of β, 1-4 linkages (Figure 2a and 2b). There is a good correspondence between the main bands of the spectra of microbial cellulose and that of MCC. The only difference between the FTIR spectra of microbial cellulose and MCC was restricted to the intensity of some bands. FTIR spectra from Figure 2b also showed that microbial cellulose is free from contaminants such as lignin or hemicellulose, which is often present in plant cellulose (Bertocchi *et al*., 1997). Such a high purity cellulose may find special applications in food or non food products.

The samples of microbial cellulose are in the polymorphic form of native cellulose similar to those obtained by plants as indicated by the position and intensity of some absorption bands in the FTIR spectra (Figure 2b). According to Nelson and O'Connor (1964a), a weak and broad band centered at 891.59 cm^{-1}, and a strong band centered at 1424.18 cm^{-1} (CH2 scissoring) were present in the spectra of the microbial cellulose samples, defining them as cellulose I. The intensity of the band at 1424.18 cm^{-1} has also been correlated with the degree of crystallinity and was often used as a standard band for its estimation (Bertocchi *et al*., 1997).

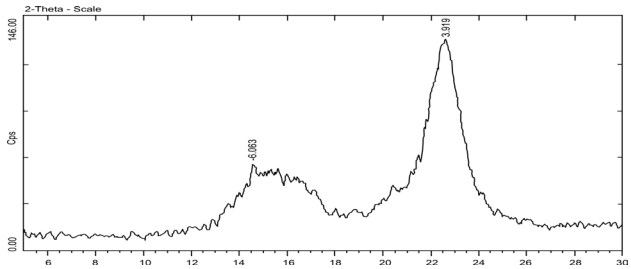
Measurement of crystallinity of cellulosic materials was performed by means of infrared crystallinity ratio (CR), given by the ratio of absorptivities at 1372 cm^{-1} (C-H bending) to that at 2900 cm^{-1} (CH_2 and CH stretching) (Nelson and O'Connor, 1964b). The infrared crystallinity ratio (CR) obtained from this study is 100 for both the microbial cellulose and the

commercial MCC. From these data, it appears that the bacterial cellulose exhibits a very high degree of crystallinity, comparable to that of MCC.

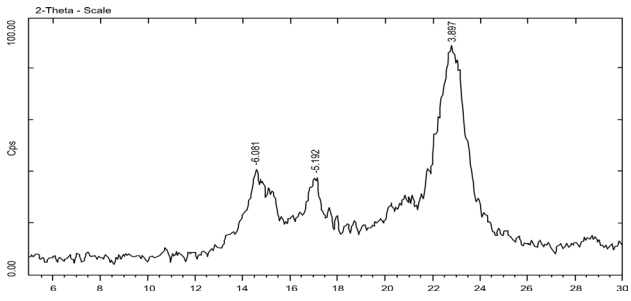
X-ray diffractometry

According to Mazumdar (1999), X-ray diffraction analysis could be used for quantitative determination of the crystalline contents in composite samples. The X-ray diffraction scans obtained for the commercial MCC is shown in Figure 3a. On the other hand, Figure 3b shows the diffractogram of the microbial cellulose. The main purpose of this study was to compare the degree of crystallinity of both the commercial MCC and the microbial cellulose. Therefore, estimation of percentage of crystallinity from these diffractograms was used to compare the integrated intensities of the crystalline peaks along with that of the diffuse background (Segal *et al*., 1959).

Findings from both the diffractograms show that the commercial MCC has a higher intensity of the crystalline phase compared to the microbial cellulose, which depicted a lower intensity of the crystalline phase. In addition, the diffraction angle at 2θ = 19°, which represents the intensity for amorphous phase for MCC also showed a higher intensity than the microbial cellulose. As a result, the overall degree of crystallinity (Cr.I.) for MCC is slightly lower than that of the microbial cellulose. The crystallinity index was 80.9 and 81.3 for MCC and the microbial cellulose, respectively.



(a)



(b)

Figure 3. (a) X-ray diffractogram of the commercial MCC, (b) X-ray diffractogram of the bacterial cellulose.

Results obtained by Bertocchi *et al*. (1997) have shown a higher degree of crystallinity as compared to the microbial cellulose produced in the present study. On the other hand, the microbial cellulose in the present study was only soaked in distilled water rather than employing non-aqueous solvents like alkyl alcohols or ketones. However, findings obtained in the present study gave a higher degree of crystallinity (Cr.I.) as compared to the cellulose which was isolated from hemp, kenaf and sorghum which only yielded 72, 65 and 63 % of Cr.I., respectively (Focher *et al*., 2001). A different content of crystalline and amorphous region in the microbial cellulose and MCC also contributed to the molecular rigidity of the bacterial cellulose fibers, which is higher than that of MCC.

Swelling properties

Cellulose is insoluble in most of the organic solvents due to its crystalline nature. However, Mantanis *et al*. (1995) have opined that both intracrystalline and inter-crystalline swelling is possible in certain solvents. According to Geyer *et al*. (1994), *Acetobacter xylinum* produces cellulose in a highly swollen form using glucose as the carbon source. The water absorption capacity of cellulose has been reported to be varying between 100 and 120 times its dry weight (White and Brown, 1989). Hence, to obtain more detailed information on the state of swelling properties, comparison was done between the microbial cellulose and the commercial MCC. The liquid retention values (LRV) of the cellulosic fibers of the microbial cellulose and MCC in four organic solvents at room temperature are shown in Figure 4.

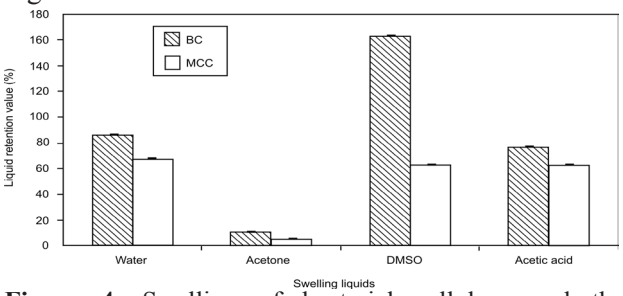


Figure 4. Swelling of bacterial cellulose and the commercial MCC in organic solvent.

As depicted in Figure 4, the microbial cellulose showed higher fiber liquid retention values (10-160%) in all the four organic solvents than that of MCC (5-70%). In addition, DMSO resulted in the highest fiber liquid retention values for both the microbial cellulose and MCC, than that of water. These results are in agreement with those reported by Mantanis *et al*. (1995). The very high swelling power exhibited by DMSO, whose molar volume is quite high, may be reflective of the very high hydrogen bonding capability.

Since Robertson (1964) suggested that molar

volume is important to swelling, water with low molar volume but with a high cohesive energy density (CED) with high hydrogen bonding capability, results in the swelling power of cellulosic fibers in water to be lower than DMSO. On the other hand, acetone exhibited the lowest fiber liquid retention values. It is likely that the hydrogen bonding capability of acetone is the weakest compared to the other three organic solvents. Apart from this, the cohesive energy density value is also found to be lowest. Therefore, these two factors might have resulted in the swelling power of microbial cellulose and MCC in acetone as the lowest. Mantanis *et al*. (1995) reported that higher the cohesive energy density of the solvent, the higher is the cellulosic fibers swelling power.

Adsorption isotherm

Besides predicting the microbial or physico-chemical stability of foods, knowledge of water adsorption isotherms is very important for engineering purposes related to concentration and dehydration. The sorption isotherms for both the microbial cellulose and the commercial MCC are shown in Figure 5.

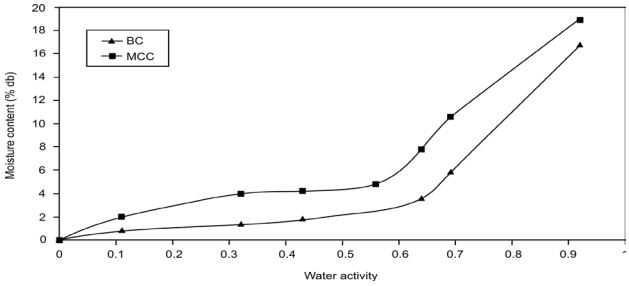


Figure 5. Moisture adsorption isotherms of bacterial cellulose and the commercial MCC at 30 °C.

It is observed that for the commercial MCC, it follows the sigmoidal-shaped curve, which might be caused by the combination of colligative effects, capillary effects, and surface-water interactions. Two bends are noted on the isotherm for MCC, one around a water activity (a_w) of 0.2-0.4 and another at 0.65-0.75, which results from changes in the magnitude of the separate physical-chemical effects. The results obtained in the present study are in accordance with the results on microcrystalline cellulose reported by Ardizzone *et al*. (1999).

On the other hand, the curve for the microbial cellulose (BC) represents the adsorption isotherm for a pure crystalline substance that shows very little moisture gain until the aw goes above the point where water begins to dissolve the crystal surface. The relative humidity where crystal dissolves is sometimes referred to as the deliquescent point. The shape of this curve is due to water interacting only via the hydrogen bonds with hydroxyl groups on the surface of the crystal. Since this is a surface effect, grinding the bacterial cellulose into smaller particle increases the moisture content at the low a_w due to the increased surface area per gram allowing more hyroxyl groups to stick out (Bell and Labuza, 2000).

As shown in Figure 5, at low a_w, for microbial cellulose, the interaction of water with cellulose molecules is not strong enough to break the interactive forces between the individual cellulose molecules in the crystal. However, as the a_w is increased, the overall water-cellulose interactions increase enough to cause disruption of the cellulose-cellulose interactions, and this water begins to penetrate into the crystal and exposing new surfaces.

For the commercial MCC, it holds more water than that of the produced bacterial cellulose, as shown in Figure 5. This happened because the less crystalline or amorphous noncrystalline material of MCC can hydrogen-bond water internally, not just on the surface. However, as the aw and moisture content increases, the cellulose molecules in the amorphous solid become more mobile as the solid becomes increasingly rubbery.

Conclusions

The characterization of microbial cellulose harvested from the tea broth revealed cellulose layer to be constituted by a compact cellulose network structure with the assembly of the ultrafine microfibrils (by SEM) and exhibited a very high degree of crystallinity at 81.3, accessed by means of FTIR, which was further confirmed by X-ray diffractometry. In addition, the microbial cellulose also depicted a high swelling power due to its structure, which easily forms hydrogen bonding as compared to the commercial cellulose. The curve for the produced microbial cellulose represents the adsorption isotherm for a pure crystalline substance. To conclude, microbial cellulose with high purity and high degree of crystallinity could be successfully produced from the fermentation of black tea broth - Kombucha.

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