

Morphological Investigation of Bacterial Cellulose/Cassava Starch Nanocomposites Produced by *In-situ* Process in Agitated Culture

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Abstract: The existence of starch in the formation of bacterial cellulose was found to have the capability to enhance the viscosity of culture medium, thus it can affect the agitation process. The aim of this study was to investigate the morphological structure of bacterial cellulose/cassava starch (BC/CS) nanocomposites produced by in-situ process in agitated culture. The fabrication of BC/CS was carried out at 28°C for 7 days under agitated condition at 100 rpm with five different masses of CS. The crystallinity of BC/CS nanocomposites was studied by using X-ray Diffraction (XRD) pattern, whereas the morphological structure was discovered from the digital photograph, optical microscope (OM) picture and scanning electron microscope (SEM) pictures. From the SEM analysis, it was observed that the cassava starch layer had occupied the pores of the fibre network of bacterial cellulose and increased the average size of the fibres. Also, it was well dispersed in the network of bacterial cellulose. The high content of starch on the culture caused the changes in orientation of the nanocomposites' surface morphology and reduced the shaking effect of the culture. The study found that the BC/CS nanocomposites with variation of 5 g CS showed the best morphological properties.

1 INTRODUCTION

Bacterial cellulose (BC), which produced by *Acetobacter xylinum*, was one of environmentally friendly products that has grown the researchers' attention nowadays due to its outstanding features, such as its higher tensile strength, crystallinity, and capacity of water absorption; besides, it has ultra-fine fibre network structure, good transparency, good chemical stability, considerable fibre binding ability, appropriate biocompatibility, biodegradability, and moldability [Ishihara et al. 2002; Klemm et al. 2001; Shezad et al. 2009; Vandamme et al. 1998]. One of its applications is as a material to solve the problem of the dependence on the petroleum products and the environmental damage [Averous, 2004; Lu et al. 2006]. As a biodegradable material, BC can be employed as a matrix of nanocomposites material.

Regarding to the above explanation, starch, which has a proper biodegradability, low cost production, and a wide availability, could be a good

filler for the matrix of bacterial cellulose to form BC/cassava starch nanocomposites [Angles and Dufresne, 2000]. The integration of those two biodegradable materials was employed since the starch itself has some drawbacks, namely, lower mechanical properties, high hygroscopicity, and high permeability to gases [Vandamme et al. 1998]. Therefore, by combining these two materials, the new biodegradable materials with enhanced properties could be yielded.

As reported by Haigler et al. (1982) the starch which is added to the culture medium of *Acetobacter xylinum* does not affect the reaction of cellulose formation even though different carbon sources, such as glucose, fructose, and gluconate, can be used to synthesize the cellulose. In other word, the starch is not consumed by the bacteria. It can be proved by the appearance of blue color on the nanocomposites after being added by iodine, which shows the existence of starch on the nanocomposites [Zhang et al. 2006; Kuipers et al. 1994]. The existence of starch on the nanocomposites of

BC/starch, furthermore, could strengthen the interaction between cellulose membrane and the wall inside the culture vessel during the inoculation process because of the high viscosity of the medium after gelatinization process of starch. That interaction, which was called as wall effect, can compress the production of cellulose by limiting the increase of cellulose membrane thickness [Homung et al. 2006]. Besides, the decrease of fluidity and the increase of viscosity due to the existence of gelatinized starch could limit the diffusion process of the glucose substrate on the cellulose matrix and the motion of *Acetobacter xylinum* in the culture medium [Yang et al. 2014].

There are two methods that are commonly used to produce the BC, namely static method and agitation method. The use of the static method on the production of BC has been proved to have some disadvantages over the agitation method. The agitation method can provide enough oxygen for the bacteria, while the static method failed to do so; thus, the agitation method can increase the production of cellulose. Also, the agitation method can reduce the crystal size and form the more stable crystal. However, based on several studies, the agitation methods can cause the stretching among the woven of cellulose fibre, thus forming the larger pores. This makes the layers of formed cellulose separated each other, so that the degree of crystallinity reduced [13–1 Watanabe et al. 1998; Yamanaka et al. 2000; Yamamoto et al. 1996].

Based on the above issues, the production of BC/starch nanocomposites using agitation method should be investigated. The agitation method was chosen because it can provide enough oxygen, while the existence of starch can reduce the shaking effect of agitation process. To date, there is no study discussed about the production of BC/starch nanocomposites using agitation method. Although there have been several research studied about the production of BC/starch nanocomposites [Yang et al. 2014; Grande et al. 2009; Martin et al. 2009; Woehl et al. 2010], those studies still used the conventional static method. This study aimed to investigate the morphological properties of BC/cassava starch (BC/CS) nanocomposites produced by in-situ process in agitated culture because it can present the effects of starch on BC visually. Thus, it can provide better understanding in the effects of starch on the formation of bacterial cellulose nanocomposites.

2 MATERIALS AND METHODS

2.1 Materials

The materials used in this study were glucose, bacto-peptone, urea, NaOH, NaOCl, CH₃COOH, and distilled water, which were purchased from Merck, without having further treatments. Coconut water was supplied from traditional markets in Medan, Indonesia and the bacteria, *Acetobacter xylinum*, was supplied by the Microbiology Laboratory of Universitas Sumatera Utara.

2.2 Isolation of Cassava Starch

The isolation of cassava (*Manihot esculanta*) starch was done by using conventional method. Briefly, the cassava was peeled. After that, it was washed using water, then shredded. The resulted cassava was added with enough water, then blended. The result was then precipitated after filtered using the gauze. The precipitation was washed frequently until the washed waste was transparent. This precipitation was called as starch. After that, it was dried in the oven at 45°C for 24 hours. The dried starch was ground and sifted in order to obtain the final starch.

2.3 Preparation of BC/CS Nanocomposite Film

BC/CS nanocomposites were produced by the *Acetobacter xylinum* bacterial strain in culture medium that containing 100 ml coconut water, 0.5% (w/v) urea, 1.0 % (w/v) glucose, 1.5% (w/v) bacto-peptone. The pH of culture medium was adjusted to 4.5 by acetic acid. CS with variations of 1 g, 2 g, 3 g, 4 g, and 5 g was added to culture medium, followed by autoclave for 30 min at 121°C. The solutions were magnetically stirred for 15 minutes. Main cultivation were carried out at 28°C for 7 days under agitated condition at 100 rpm. The BC/CS nanocomposites were washed under running tap water and it was immersed overnight in 2.5% NaOH and also in 2.5% NaOCL. Then, it was rinsed again under running tap water to remove any solvent until it reached neutral pH. The BC/CS nanocomposites were finally pressed using hot-press with wire-mesh at 115°C for 10 min.

2.4 Characterization

The BC/CS nanocomposites were characterized by X-ray diffraction (XRD), scanning electron

microscope (SEM), and optical microscope (OM). The XRD pattern were taken by Shimadzu XRD-6100 diffractometer using Cu-K α radiation ($\lambda = 0.154$ nm) at scanning rate of 2°/min, a voltage of 40kV and a current of 200mA. The diffraction angle (2θ) range from 5° to 30° with a step size of 0.02°. The degree of crystallinity (CrI) was calculated from diffracted intensity data using the method described by previous researcher (Seagel et al., 1959) as shown on the formula (1)

$$CrI (\%) = (1 - (I_{AM} - I_{200})) \times 100\% \tag{1}$$

Where the I_{AM} and I_{200} represented the intensity of diffraction in the same units at approximately $2\theta = 18^\circ$ and maximum intensity of (002) lattice diffraction at approximately $2\theta = 22.7^\circ$, respectively.

The scanning electron microscope was done using SEM EDX EVO MA 10 Carl Zeiss Bruker. All samples were sputter coated with gold-palladium and observed using an accelerating voltage 20 kV. Samples were viewed at magnification between 1000 and 10000 times from their original sizes. The optical microscope pictures were taken using American Optical Microscope with the magnification of 100 times

3 RESULT AND DISCUSSION

3.1 XRD Analysis

As shown in Figure 1, the diffraction peak of starch was found at 2θ of 14,780°, 16,924°, and 22,040°. This is in agreement with the results reported by Grande et al. [Grande et al. 2009]. The starch showed lower crystallinity value. This was caused by two conditions from the gelatinization process that occurred during the formation of nanocomposites. As gelatinized CS, the crystal from starch granules was damaged and the intensity related to the peak of diffraction will decrease, or even disappear. The crystallinity of starch was equal to zero because there are no crystal peaks observed on the spectra.

The diffraction peaks for BC was discovered at 2θ of 14.208°, 16.930°, and 22.924°, which showed the diffraction lattice of (1 $\bar{1}$ 0), (110), and (002), respectively, at the polymorph of cellulose I. This result is in accordance with the result reported by

Table 1. Diffraction peaks, d-spacing, and degree of crystallinity of CS, BC, and CS/BC nanocomposites.

Sample	2θ (°)	d (nm)	Degree of crystallinity (%)
CS	15.030°	5.889	~0%
	17.880°	4.956	
	23.173°	3.835	
BC	14.827°	5.969	92%
	17.362°	5.103	
	23.168°	3.836	
BC/CS	14.208°	6.228	53%
	16.930°	5.233	
	22.924°	3.876	

Yang et al. 2014. As also can be seen from figure 1, the BC has sharp peaks because of the high crystallinity degree of it (92%, shown in table 1). It can be explained as a result of the intermolecular hydrogen bonding in the cellulose structure.

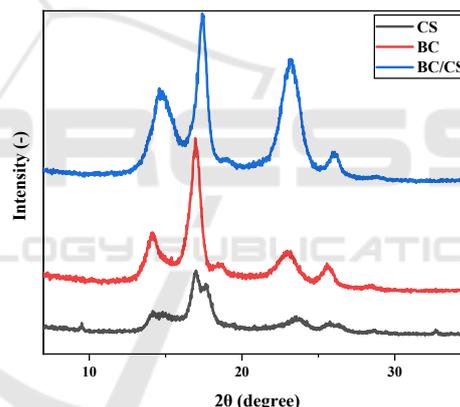


Figure 1. X-ray diffraction spectra of CS, BC, and CS/BC nanocomposites.

For the BC/CS nanocomposites, the diffraction peaks was placed at 2θ of 14.827°, 17.362°, and 23.168°. This result is also similar to the results obtained by Yang et al. (2014). As previously mentioned that the BC itself has a high degree of crystallinity, the existence of cassava starch, furthermore, reduce its degree of crystallinity to 53% as shown in table 1. The decrease in degree of crystallinity of BC/CS nanocomposites occurred due to two possible reasons: 1. The migration of *Acetobacter xylinum* was blocked by the poor fluidity of medium due to the existence of gelatinized starch. In other word, the motion of bacteria was limited; 2. There was a high steric obstruction in amylopectin branching on that prevent

the formation of cellulose bands since the amylopectin stick in the cellulose microfibrils [Yang et al. 2014]

3.2 The Morphology of BC/CS Nanocomposites Analysis

Figure 2 shows the digital photograph of the BC membrane and the BC/CS membrane with various mass of CS after 7-day agitation process. It can be seen that the mass of CS affects the layer yielded, the more CS added, the more organized the BC/CS nanocomposites obtained. The BC/CS nanocomposites with the variation of 5 g CS show the most organized one. It is similar to the BC/CS nanocomposites produced by static method. This can be occurred because the starch can increase the viscosity of culture medium, thus it can reduce the shaking effect during the agitation process.

The optical microscope pictures from BC and BC/CS nanocomposites with the variation of 5 grams of CS in never-dried state were given in figure 3. As seen in figure 3(a), the surface of BC is more transparent than that of BC/CS nanocomposites. The surface of BC reveals the fibre network of the BC, while the surface of BC/CS nanocomposites looks solid without having any fibre (figure 3(b)). This happened because the pores of BC have been filled by the swollen starch granules.

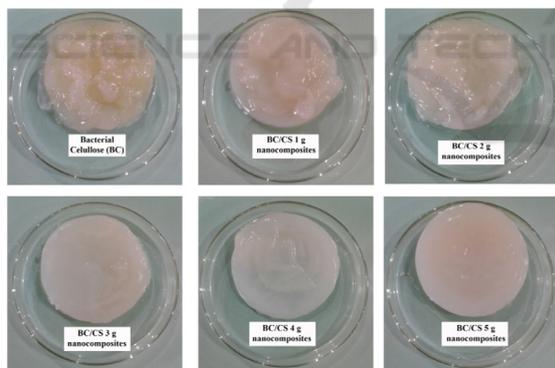


Figure 2. Digital photos of pure BC and BC/CS nanocomposites with various mass of starch with magnitude (a) 100x in never-dried state.

The surface morphology of CS, BC and BC/CS nanocomposites is also further investigated through SEM with 10,000 times of magnification and is shown in Figure 4. Figure 4(a) gives the shape of starch granules clearly with a perfect oval shape. Based on our analysis, the average size of them is around 12.755 μm . The large size of the granules indicates the high capability of capping the water during the gelatinization process.

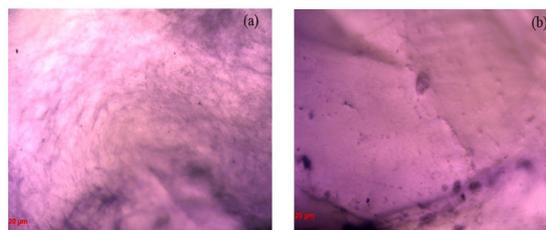


Figure 3. Optical microscope images of BC and BC/CS nanocomposites with magnitude (a) 100x in never-dried state

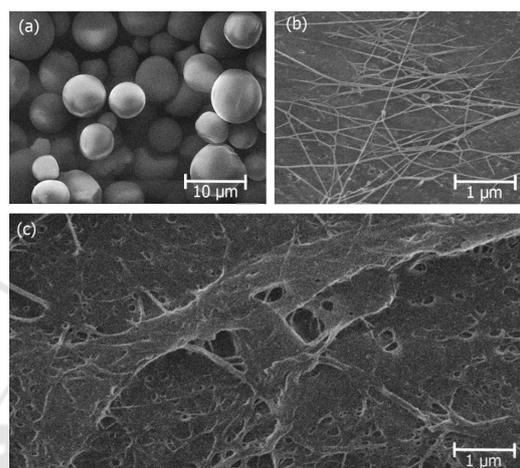


Figure 4. SEM images of (a) CS, (b) BC, and (c) BC/CS nanocomposites with magnitude 10,000x after hot-pressing.

Figure 4(b) indicates the irregularity of BC network obtained by agitation method. This can be occurred because during the process, the bacteria move to the oxygen-rich area. The agitation method will cause the stretching happened among the woven of cellulose fibre and result in larger pores among them. Still, this method can reduce the size of fibre or the size of crystal [Watanabe et al.1998].

The two above results formed another structure when they combined as BC/CS nanocomposites due to the interaction between them. This is shown in Figure 4(c). The gelatinized CS was dispersed evenly in the matrix of BC. The high content of starch caused the integration of BC network and gelatinized starch; the starch did not only stick in the BC fibre, but also resulted in the changes in the orientation of nanocomposites' surface morphology. Based on the SEM analysis, it was discovered that the BC has average size of fibre of 67.907 nm, while BC/CS nanocomposites have the average size of fibre of 73.470 nm. The increase of fibre size occurred because the shaking effect decreased after the addition of starch.

4 CONCLUSION

In this study, BC/CS nanocomposites were successfully obtained by *in-situ* process in agitated culture. The XRD pattern shows that the degree of crystallinity of BC/CS nanocomposites was lower than that of the BC. From the digital photograph, OM and SEM pictures, it was proven that the existence of starch could rectify the irregularity of cellulose layer produced by agitation method. The gelatinized CS is well dispersed in the network of BC and filled the pores of the BC fibre, thus increases the average size of the nanocomposite fibres. Also, it causes the changes in the orientation of nanocomposites' surface morphology. The more CS added, the more organized layer yielded. This can be occurred because the increase gelatinized starch can raise the viscosity of culture medium and reduce the shaking effect. Furthermore, it was found that the BC/CS nanocomposites with the variation of 5 g CS performed the best result.

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REFERENCES

- Anglès, M. Neus, and Alain Dufresne. 2000. "Plasticized Starch/Tunicin Whiskers Nanocomposites. I. Structural Analysis." *Macromolecules*.
- Avérous L, Boquillon N. 2004. "Biocomposites Based on Plasticized Starch: Thermal and Mechanical Behaviour." *Carbohydrate Polymers*.
- Czaja, Wojciech, Dwight Romanovicz, and R. malcolm Brown,. 2004. "Structural Investigations of Microbial Cellulose Produced in Stationary and Agitated Culture." *Cellulose*.
- Grande, Cristian J. et al. 2009. "Development of Self-Assembled Bacterial Cellulose-Starch Nanocomposites." *Materials Science and Engineering C*.
- Hornung, M., M. Ludwig, A. M. Gerrard, and H. P. Schmauder. 2006. "Optimizing the Production of Bacterial Cellulose in Surface Culture: Evaluation of Substrate Mass Transfer Influences on the Bioreaction (Part 1)." *Engineering in Life Sciences*.
- Ishihara, M, M Matsunaga, N Hayashi, and V Tisler. 2002. "Utilization of D-Xylose as Carbon Source for Production of Bacterial Cellulose." *Enzyme Microb Technol*.
- Klemm, Dieter, Dieter Schumann, Ulrike Udhardt, and Silvia Marsch. 2001. "Bacterial Synthesized Cellulose - Artificial Blood Vessels for Microsurgery." *Progress in Polymer Science (Oxford)*.
- Kuipers, AGJ., E. Jacobsen, and RGF. Visser. 1994. "Formation and Deposition of Amylose in the Potato Tuber Starch Granule Are Affected by the Reduction of Granule-Bound Starch Synthase Gene Expression." *The Plant cell*.
- Lu, Yongshang, Lihui Weng, and Xiaodong Cao. 2006. "Morphological, Thermal and Mechanical Properties of Ramie Crystallites—reinforced Plasticized Starch Biocomposites." *Carbohydrate Polymers*.
- Martins, Ivo M.G. et al. 2009. "New Biocomposites Based on Thermoplastic Starch and Bacterial Cellulose." *Composites Science and Technology*.
- Shezad, Omer, Salman Khan, Taous Khan, and Joong Kon Park. 2009. "Production of Bacterial Cellulose in Static Conditions by a Simple Fed-Batch Cultivation Strategy." *Korean Journal of Chemical Engineering*.
- Vandamme, E.J. et al. 1998. "Improved Production of Bacterial Cellulose and Its Application Potential." *Polymer Degradation and Stability*.
- Watanabe, Kunihiko, Mari Tabuchi, Yasushi Morinaga, and Fumihiko Yoshinaga. 1998. "Structural Features and Properties of Bacterial Cellulose Produced in Agitated Culture." *Cellulose*.
- Woehl, Marco Aurélio et al. 2010. "Bionanocomposites of Thermoplastic Starch Reinforced with Bacterial Cellulose Nanofibres: Effect of Enzymatic Treatment on Mechanical Properties." *Carbohydrate Polymers*.
- Yamamoto, Hiroyuki, Fumitaka Horii, and Asako Hirai. 1996. "In Situ Crystallization of Bacterial Cellulose II. Influences of Different Polymeric Additives on the Formation of Celluloses Ia and Ib at the Early Stage of Incubation." *Cellulose*.
- Yamanaka, S, M Ishihara, and J Sugiyama. 2000. "Structural Modification of Bacterial Cellulose." *Cellulose*.
- Yang, Jingxuan et al. 2014. "In Situ Fabrication of a Microporous Bacterial Cellulose/Potato Starch Composite Scaffold with Enhanced Cell Compatibility." *Cellulose*.
- Zhang, Qingmin et al. 2006. "Direct Detection of the Formation of V-Amylose Helix by Single Molecule Force Spectroscopy." *Journal of the American Chemical Society*.