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NOVEL COMPOSITE OF SILVER-BACTERIAL CELLULOSE (Ag-BC) FROM SIWALAN SAP (*Borassus flabellifer*) AND ITS ANTIBACTERIAL ACTIVITY

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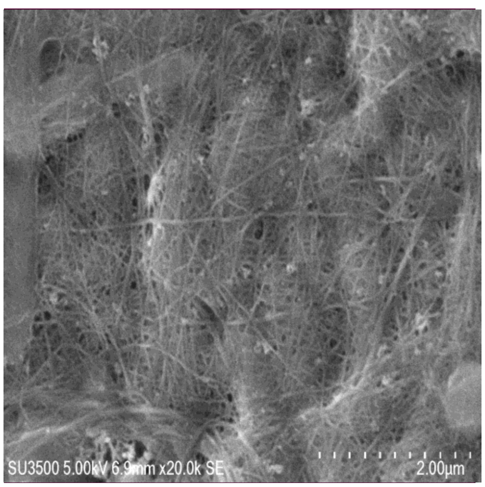
Graphical abstract



Abstract

Recently, a wound healing from natural composite with excellent properties is in a high demand. In this study, a novel composite of bacterial cellulose made from Siwalan sap (*Borassus flabellifer*) was achieved. Siwalan is a common plant in Java Island of Indonesia and the application is very limited for beverage only. This study aims to determine the effect of the $AgNO_3/NaBH_4$ concentration ratio in the development of Ag-BC composites and its antibacterial properties from Siwalan sap. Ag-BC composites were prepared by impregnating the silver solution into the BC matrix through the reduction process with $NaBH_4$. Characterization of Ag-BC composites conducted using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), and Energy Dispersive X-ray (EDX). Antibacterial assay was performed using disc diffusion method against *Salmonella typhimurium* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) bacteria. The characterization of Ag-BC composite shows the nanostructure of BC with a length of fiber around 35-60 nm in width. The SEM-EDX micrograph showed that silver particles were impregnated into the BC matrix. Antibacterial activity test results showed that the Ag-BC composite had the ability to inhibit the bacteria *S. typhimurium* and *S. aureus* with good inhibition. This result showed the potential application of Ag-BC composite from Siwalan plant as a natural material for medical and pharmaceutical purpose, especially as an antibacterial agent.

Keywords: Bacterial cellulose, silver, composite, antibacterial activity, *Borassus flabellifer*

1.0 INTRODUCTION

Silver ions and silver compounds have toxic properties in some bacteria, viruses, algae, and fungi such as silver nitrate and sulfadiazine. Some researchers reported that silver particles can attack bacterial cells causing cell damage and killing the bacteria [1-5]. Silver particles in the form of nanoparticles (AgO), oxides or in the form of ions (Ag^+) have a good antibacterial ability. The smaller the particle size of silver, the higher the ability of it to kill bacteria, and because of that, the properties of silver particles are utilized in the biomedical field [6-8].

In the biomedical field, in addition to utilize silver particles, bacterial cellulose (BC) material is important as raw support material. BC is a natural polymer of extracellular polysaccharides consisting of long non-aggregate nano fibrils produced by several Gram negative bacteria such as *A. xylinum* [9-11]. BC is a material that has high biocompatibility properties and does not cause allergic reactions. Moreover, even though BC does not have antibacterial properties, it can be used as support material in biomedical field such as wound dressings for burns and ulcers, regenerative skin therapy, artificial blood vessels, and tissue engineering [12-14].

Siwalan is common plants in Java Island of Indonesia and the utilization is limited for beverage only. Siwalan sap is water that comes out of the Siwalan tree through flower stalks that are cut or tapped. Siwalan sap has a sweet taste, a little sour, and has a distinctive taste. However, it only is able to be consumed fresh for 3-4 hours only. Beyond that limit, Siwalan sap immediately can turn into wine or alcoholic drinks, which have a bitter taste and can be intoxicating because it has high alcohol content [15]. The composition contained in Siwalan sap is 85.7% water; 0.38% protein; 14.38% sucrose; 0.1% fat; 0.27% ash material. Sucrose levels of Siwalan sap is high enough so that Siwalan sap can be used as a biocellulose material as it is without the addition of sugar.

Since Indonesia has a lot of Siwalan sap in an abundant amount, this research focuses on overcoming the problem of Siwalan sap' shelf life by converting it as a raw material for producing BC. To date, there has been a number of researches that study Siwalan sap as bacterial cellulose. To our knowledge, this was the first study on impregnation of silver ion to BC cellulose from Siwalan sap to evaluate its antibacterial properties. The purpose of this study was to produce novel composite of Ag-BC from Siwalan sap and characterize it. The antibacterial activity was performed as it would be potentially applicable as wound dressing in the fields of biomedical or pharmaceutical.

2.0 METHODOLOGY

2.1 Materials

The materials used in this study were Siwalan sap obtained from Tuban district, East Java province, Indonesia. $NaBH_4$, $AgNO_3$, and Nutrient Broth (NB), Nutrient Agar (NA), and NaCl were from Sigma-Aldrich. *Acetobacter xylinum* FNCC 001, Stapylococcus *aureus* FNCC 0047, and *Salmonella typhimurium* FNCC 0165 were obtained from PAU Laboratory, Gadjah Mada University, Yogyakarta, Indonesia.

2.2 Media Culture and BC Production

The BC production referred to the research of Almeida *et al.* (2014); Indrianingsih *et al.* (2017) [16, 17a] with little modifications. The media consisted of 200 ml of Siwalan sap with the addition of 1 gram of urea. The pH of Siwalan sap was 4.5 so it does not need pH adjustment for *A. xylinum* to grow. The sap media was sterilized at 121°C temperature using autoclave for 15 minutes. BC production was carried out by adding 10 % of the *Acetobacter xylinum* inoculum into each petri dish that has been filled with 10 ml of Siwalan sap media. The mixture was then incubated at room temperature for 4-5 days to form BC pellicles. The resulting BC was then harvested and boiled in distilled water for 60 minutes and rinsed in running water until pH neutral. The drying process of BC was conducted at room temperature for 24 hours.

2.3 Impregnation of Silver into Biocellulose

The impregnation of silver ions into BC pellicles were conducted by immersing BC into silver solution with several concentrations (10^{-4} M; 10^{-3} M; 10^{-2} M) for 90 minutes and then, washed with 96% ethanol for 30 seconds in order to remove the remnants of nitrate compounds. The mixture was added to $NaBH_4$ solution for 10 minutes and then, washed again with distilled water for 10 minutes. The variation of $AgNO_3/NaBH_4$ ratio was 1:5; 1:50; and 1:500. It was dried for 24 hours at room temperature (approximately 28-30°C).

2.4 Characterization of Pure BC and Ag-BC Composite

Characterization of pure BC and Ag-BC composites was conducted using FTIR and SEM-EDX. The morphological structure of pure BC and Ag-BC composites were examined on Scanning Electron Microscope SEM (SU-3500 Hitachi). The acceleration voltage of work was operating at 15 kV with a magnification of 10,000. FTIR spectra to evaluate the functional groups of pure BC and Ag-BC composites were recorded on 8201PC Shimadzu, Japan at a frequency range of 4000-400 cm^{-1} with resolution of 4 cm and 32 scans for each sample.

2.5 Swelling Measurement

This swelling percentage of Ag-BC was determined using method by Maneerung *et al.* (2007) and Indrianingsih *et al.* (2017) [11, 17a] with little modifications. Ag-BC composites and pure BC as a control medium were cut into a size of 0.5 cm x 0.5 cm in length and immersed in the distilled water for 3 days or 72 hours. Swelling capacity was calculated as follows:

Swelling = ((Gs-Gi)) / Gi

where Gi is the initial weight of dried sample and Gs is the weight of sample in swollen state.

2.6 Antibacterial Assay

The antibacterial activity test was carried out by disc diffusion method with slight modification from the literatures [11, 12,17a, 18]. The Ag-BC composite where cut in a circle shape with a diameter of ± 10 mm, then it was sterilized using an autoclave at 121°C for 15 minutes. Ag-BC composite was placed on the NA surface in a petri disk filled with 100 µL suspensions of pathogenic bacteria (*S. aureus, S. typhimurium*) with concentration of 0.9×10^9 CFU/ml and then, the media were incubated at 37 °C for 24 hours. The inhibitory activity of Ag-BC was confirmed by measuring the clear zone formed around the Ag-BC composite.

3.0 RESULTS AND DISCUSSION

3.1 Physical Appearance of Pure BC and Ag-BC Composites from Siwalan Sap

BC was produced from Siwalan sap taken from the Tuban area, East Java. The resulted BC with a diameter 9 cm had an average weight of 12.4 g and has a thickness of 1.8 mm. The water content of BC was 95.03 %. The image of pure BC and Ag-BC composites produced from Siwalan sap can be seen in Figure 1.

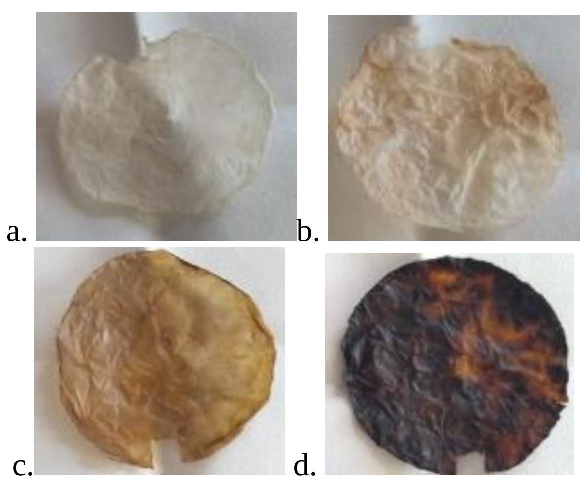
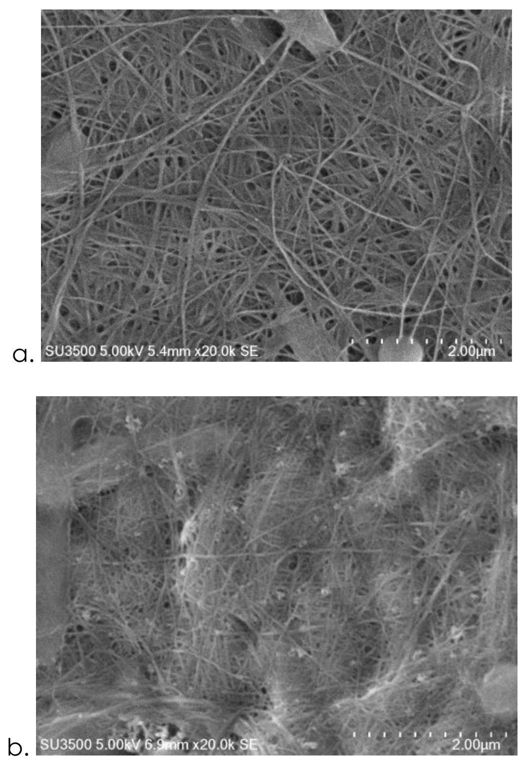


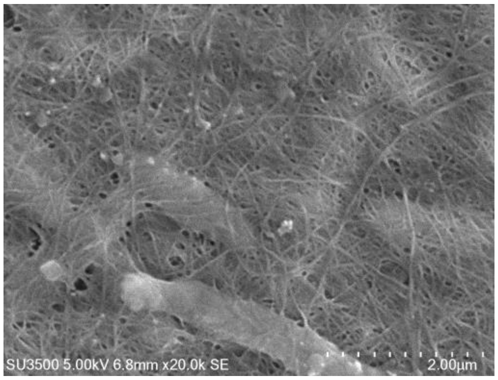
Figure 1 BC pellicles from Siwalan sap (a) pure BC, (b) Ag-BC composite with $AgNO_3/NaBH_4$ 1:500, (c) Ag-BC composite with $AgNO_3/NaBH_4$ 1:50, (d) Ag-BC composite with $AgNO_3/NaBH_4$ 1:5

The process of impregnation of silver particles in the BC matrix was by penetrating of silver ions into the biocellulose matrix through the pores. Ag^+ was absorbed by the matrix and was bound to the part of the BC microfibrils probably by ionic bonds. The presence of hydroxyl groups in BC made electron-rich oxygen atoms interact with Ag ion. The presence of ether groups in BC was expected to interact with electropositive transition metal cations or Ag^+ [11, 13, 19]. The rinsing process of Ag-BC with ethanol could remove Ag^+ which was not bound to the BC matrix. After the reduction process with 0.05 M of $NaBH_4$, silver ions are reduced to form Ag-BC composites. After the Ag reduction process, it would turn to black brown and yellow brown. The brownish yellow color indicated the formation of Ag-BC composites. After the reduction process, Ag-BC was dried at room temperature.

3.2 Surface Morphology of Pure BC and Ag-BC Composites from Siwalan Sap

The morphological surface characterization using SEM of the pure BC pellicles showed that BC consisted of nanofibrils and formed a porous structure as shown in Figure 2. The 20-50 nm nanometer-sized fibers were interconnected to form a porous structure like finely woven. Figure 2 showed the presence of bacterial cells from BC in the matrix. Bacterial surfaces consist of many strings bound together to produce an aggregate structure.





c.

d.

Figure 2 SEM photographs of (a) pure BC, (b) Ag-BC composite with $AgNO_3/NaBH_4$ 1:500, (c) Ag-BC composite with $AgNO_3/NaBH_4$ 1:50, (d) Ag-BC composite with $AgNO_3/NaBH_4$ 1:5

The surfaces of BC structure composed of many intertwined string neat fibrils. The results of BC studies of Siwalan sap were in accordance with existing BC studies, such as literatures that reporting fine fibrils that overlap one another as layers as of cellulose tape [17b, 20].

Figure 2 (b-d) shows Ag-BC composite morphology. The results of the analysis of BC impregnated with silver particles with different molarity ratios of $AgNO_3/NaBH_4$ were seen by the deposition of silver particles in the BC matrix. Agglomeration occurs because silver particles accumulate during the impregnation process causing the shape and size of silver particles to differ. Agglomeration causes silver particles to lose properties related to nano size. The degree of agglomeration becomes an important parameter in toxicology tests [21]. This possibly influences the antibacterial activity test in which the Ag-BC composite has not yet been optimized in inhibiting the bacteria *S. aureus* and *S. typhimurium*. From the results of the analysis of Figure 2, it can be concluded that the morphology of BC before being impregnated with silver is very different. The results of the previous study revealed several key factors that determine the structure of Ag-BC composites such as Ag particle size, Ag concentration in Ag-BC, the method used to produce silver nanoparticles, and techniques for inserting silver nanoparticles into the BC film [22]. EDX analysis is also needed in research to determine the composition of Ag-BC composites.

EDX analysis of the sample of pure BC and Ag-BC composites from Siwalan sap was conducted to determine the composition of its chemical elements. The results of the EDX analysis were presented in Table 1. It was observed that the microstructure of pure BC from the Siwalan sap contains C and O. This was because the structure of BC consisted of glucose monomers that form chains with other glucose through glycoside bonds (1,4 D-glucopyranose anhydride) which had intra-molecular and intermolecular hydrogen bonds and van deer Waals bonds.

Table 1 Result analysis SEM-EDX of pure BC and composite Ag-BC

Molar ratio $AgNO_3:NaBH_4$	Element (%)				
	C	O	Na	Ag	Total
0:0	47.26	52.74	-	-	100
1:500	47.67	50.34	1.99	-	100
1:50	49.2	42.22	6.13	2.45	100
1:5	45.54	47.34	3.64	3.48	100

The results of SEM-EDX analysis on Ag-BC composite samples indicated that the impregnation process did not occur perfectly. In Ag-BC composite with $AgNO_3/NaBH_4$ ratio of 1:500, the silver particles were not detected on the EDX measurements. However, there was a physical change of colour (Figure 1b). It was an indication that small amount of silver is attached; however, for the reason of the limited detection of the instruments, it was not detected. That happened because when immersing BC into silver solution, silver ions were unable to penetrate the BC membrane so that at the stage of washing with 96% ethanol solution the silver particles were lost, and the reduction process was not optimal. The reducing agent $NaBH_4$ would release Na^+ and would stick to the BC membrane. The amount of reducing agent concentration also affected the formation of silver particles.

3.3 FTIR Characterization of Pure BC and Ag-BC Composites

The results of the FTIR characterization of pure BC and Ag-BC composites from Siwalan sap were presented in Figure 3.

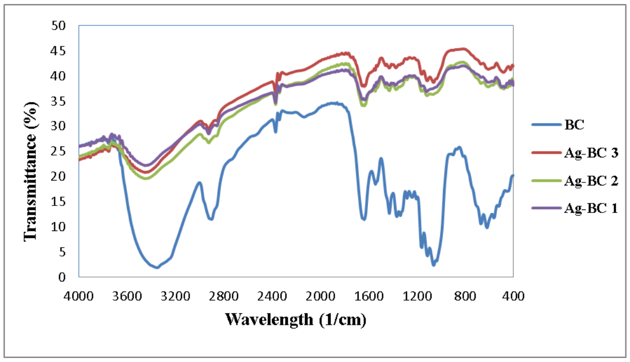


Figure 3 FTIR Spectra of pure BC and Ag-BC composites from Siwalan sap

In pure BC, it is known that the absorption of O-H groups at number 3348.42 cm^{-1} and the C-O-C group at a wavelength of 1056.99 cm^{-1} shows the presence of glycoside bonds and C-O-C in the cellulose ring. At a wavelength of 2900.94 cm^{-1} there is a C-H stretching and 1427.32 cm-1 there is a bending C-H bond. The absorption of the C = O group is at a wavelength of 1675.64 cm-1. All of the peak absorption of BC from Siwalan sap is almost the same as BC, which has been produced from coconut water by literatures [20].

From the FTIR Spectra in Figure 3, in the wavelength range of 3387-3500 cm^{-1} there is O-H stretching of hydroxyl groups that exist in the BC tissue. There is a band at a wavelength of 2877 cm^{-1} with vibrations of C-H and CH_2 groups. The absorption peak at 1064 cm^{-1} is the stretching of C-O-C groups from the BC matrix. In this study, O-H group absorption occurs at a wavelength of 3348.42 cm^{-1} which has the most extensive area. However, in the Ag-BC composite the area of the O-H group band was changed, this is possibly due to the presence of silver ions bound to the BC matrix so that the O^- group will bind to Ag^+ [23].

The band of C-H groups in pure BC and Ag-BC composites bears similarity at wavelengths of 2854.65 cm^{-1}, and 2862.36 cm^{-1}. The absorption peak at 1635.64 cm^{-1} is indicating the presence of the C = O group. The absorbance of functional group observed in pure BC and Ag-BC composites were the same as the previous studies of literatures [23, 24].

3.4 Swelling Capacity

Swelling test on Ag-BC composites with different $AgNO_3/NaBH_4$ ratios in this study can be seen in Figure 4.

Capability of pure BC on swelling is the highest compared to Ag-BC composites. BC is a hydrophilic material which is expected to be able to absorb high amount of water. BC has three-dimensional tissue with many pores. Based on the results of the swelling it can be seen that the process of impregnation of silver into BC was successful. It means that the silver particles are bound to BC, thus preventing Ag-BC composites from absorbing more water compared to pure BC. Ag-BC composites are important ingredients in making wound dressings to maintain the level of wound moisture [17b].

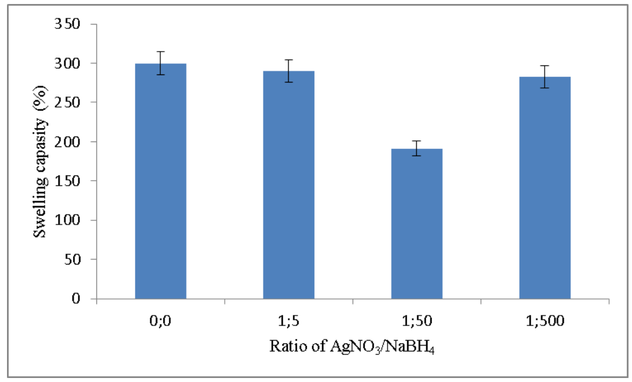


Figure 4 Swelling capability of Ag-BC composite

3.5 Antibacterial Activity of Ag-BC Composites

The reaction time for synthesis of Ag-BC composites was 90 minutes of impregnation time. The results of antibacterial activity tests of Ag-BC composites have been presented in Table 2.

Table 2 Results of Ag-BC composite antibacterial activity test

Ratio $AgNO_3:NaBH_4$ (M)	Ag-BC composite inhibition zone (mm)	
	S. aureus	***S. typhimurium***
0:0	-	-
1:5	2.31	2.58
1:50	1.92	2.03
1:500	-	0.75

Based on the results of antibacterial activity in Table 2 it can be concluded that Ag-BC composite can inhibit the growth of *S. typhimurium* and *S. aureus* bacteria. Pure BC (ratio 0:0) as a control sample showed no inhibition against *S. aureus* and *S. typhimurium*. The Ag-BC composite of 1:500 is only able to inhibit *S. typhimurium*. Ag-BC composites with a concentration ratio of $AgNO_3/NaBH_4$ (1:50) showed the presence of silver particles impregnated on the BC membrane. In the Ag-BC composite of ratio 1:50 the silver concentration absorbed 2.45 %, while Na concentration absorbed 6.13%. Ag-BC composites with concentration of $AgNO_3/NaBH_4$ ratio of 1:5, showed the highest inhibitory zone against *S. aureus* and *S. typhimurium*. In this composite, the Ag^+ concentration absorbed 3.48%, while Na^+ as much as 3.64%. Because the concentration of Ag^+ absorbed in BC is lower than the concentration of Na^+, a cation concentration gradient occurs, Na^+ will penetrate into the BC, and Ag^+ does not penetrate out [11, 17b].

4.0 CONCLUSION

Ag-BC composites from Siwalan sap had been made with various molarity ratios and had good swelling ability and antibacterial activity. The higher the concentration of silver particles, the higher the chance of formation of aggregation in particles. The ratio of $AgNO_3/NaBH_4$ in Ag-BC composite which has the ability of antibacterial activity is (1: 5) and (1:50). Antibacterial activity test results showed that the Ag-BC composite had the ability to inhibit the bacteria *S. typhimurium* and *S. aureus* with good inhibition. This result showed the potential application of Ag-BC composite from Siwalan plant as a natural material for medical and pharmaceutical purpose, especially as an antibacterial wound dressing.

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