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CHARACTERIZATION AND THE ACTIVITY OF BACTERIAL CELLULOSE PREPARED FROM RICE WASTE WATER BY ADDITION WITH GLYCEROL AND CHITOSAN

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ABSTRACT

Bacterial cellulose (BC) and its composites were synthesized from rice waste water (RWW) by addition of glycerol (G) and chitosan (Ch). The BC, BC-G and BC-G-Ch were characterized by several methods including Fourier Transform Infra Red (FTIR), Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), Thermogravimetric Analysis and Differential Thermal Analysis (TGA-DTA) and tensile tester. The data of FTIR, XRD, and TGA-DTA confirmed the presence of glycerol and chitosan in BC composites. The antibacterial activity of BC and its composites were tested against *Staphylococcus aureus* ATCC 25923 by clear zone method. The experimental data shows that BC-G-Ch exhibits a higher antibacterial activity in comparison to BC and BC-G.

Keywords: *Acetobacterxylinum*, antibacterial activity, bacterial cellulose, chitosan, glycerol.

INTRODUCTION

Cellulose is anabundant natural polymer material which is produced naturally by many plants, algae, and also an assortment of bacteria [1]. Agrobacterium, Rhizobium, Pseudomonas, Sarcina, and Acetobacter are some genera of bacteria which produce cellulose [2]. The BC which is produced by *Acetobacter xylinum,* shows significant difference from cellulose obtained from plants. A network of pure cellulose is free lignin and hemicellulose which canbe obtained in a culture medium as a pellicle composed of fibers with a size less than 100 nm. These cellulose fibers are composed of fine threads nanoscale [1,2]. Commercial product of cellulose can be used as an ingredient for making tires, membranes of headphones, special paper, textile, and biomedical applications as a temporary skin substitute [2] derived from nanometer-sized cellulose with unique properties.

The purity of bacterial cellulose is higher than that of cellulose of plants, and it does not contain lignin and hemicellulose. BC is obtained through a simple process, no further purification process to remove contaminants and unwanted polymer, and therefore, it retains a greater degree of polymerization [3]. The BC presents in high christalinity. Cellulose I and cellulose II are two crystalline forms of cellulose. *A. xylinum* in static culture and most of the plants produce cellulose I. Cellulose I and cellulose II are composed of β-1,4-glucan chains in different structure. Cellulose I exhibits parallel structure, whereas cellulose II exhibits random and antiparallel structure, and also hydrogen bonds, so that the cellulose II shows a higher thermodynamic stability than cellulose I [1, 4]. The BC is also used as dietary fiber and as a binding or thickening agent [1]. Crystallinity and the amount of acrylic that can be grafted into the cellulose structure will affect the ability of BC to absorb water. Biomedical material has been studied from a cellulose phosphate membrane. Similarly, bone regeneration applications and osteo integration have been studied from phosphorylated cellulose [2].

The BC exhibits aunique structure and mechanical properties in comparison with higher plant cellulose. A surface area per unit mass of the BC fiber is high, as a result of its diameter of 20-100 nm. The combined properties of cellulose has a large surface area with highly hydrophilic property, and the resulting material can be applied in various fields with a very wide area, particularly in biomedical needs and biotechnology [5]. It is important to design an efficient production of BC by aeration and agitation method, so it can be produced effectively with good quality and economical for industrial-scale production [6]

Bacterial cellulose can be prepared from household waste, such as RWW. The RWW contains starch, protein, minerals, and vitamin B, which can be used as a source of nutrients for *Acetobacter xylinum* in synthesizing bacterial cellulose. Vitamin B in RWW will help *Acetobacter xylinum* to grow in the bad environment. Glucose in RWW is used by bacteria in fermentation process, while protein in RWW can be used as a source of nitrogen, and sucrose was added in the substrate of RWW as a source of carbon which is needed by *Acetobacter xylinum*. Urea can be used as an additional source of nitrogen also.

Medical applications of BC primarily aim to prevent aninfection by pathogenic microorganisms. Addition of chitosanon BC is aimed to provide antimicrobial properties to cellulose. Chitosanis aglucosamine biopolymer produced from deacetylation of chitin. Recent studies claimed that a bacterial growth can be inhibited effectively by chitosan [7, 8]. Chitosanhas a positively charged amine groups that will bind to the negatively charged groups on the surface of microorganisms [8]. Institute of Chemical Fibers (ICWH) Poland has synthesized BC composite by introducing chitosan on BC [8].

According to The National Nosocomial Infection Surveillance System (NNIs), *Staphylococcus aureus* is one of the most common pathogens in the wound. Wei *et al*. [9] stated that *Staphylococcus aureus* is one of gram-positive bacteria generally found on the contaminated wounds. Due to that reason, we focused on preparation of BC and its composites from RWW on addition with glycerol and chitosan and its application as antibacterial materials against *Staphylococcus aureus*.

MATERIALS AND METHODS

Rice was purchased from the traditional market in Yogyakarta. The RWW was obtained from the first wash of rice by aquadest. Sucrose, urea, glycerol, and acetic acid were purchasedas commercial products and used as they were without any further purification. Nutrient Agar (NA) and Nutrient Broth (NB) were purchased from Oxoid. *Acetobacter xylinum* and chitosan (DD = 73.78%) were purchased from Bratachem, Yogyakarta and *Staphylococcus aureus* was obtained from collection of Faculty of Medicine, Universitas Gadjah Mada.

Preparation of bacterial cellulose and its composites

Rice wastewater was prepared by washing a kilogram of rice by a liter of aqua dest. The mixture was filtered and the filtrate was collected in a container. The BC-G-Ch was prepared as the following. Twenty grams of sucrose, 1.0g of urea, and 1.0 g of glycerol were mixed with 200mL RWW. The mixture was poured intoan Erlenmeyer and stirred magnetic ally until dissolved and the pH of solution was set to 3-4 by adding glacial acetic acid. Subsequently, the mixture was poured into a tray and allowed to cool to room temperature. *Acetobacterxylinum* (40mL) was then introduced into the solution and fermented for7-14daysat room temperature. The formed pellicle layer was washed several times with tap water, distilled water and hot water, respectively. Then 2 wt.% of chitosan solution with deacetylation degree of 73.78% was poured onto the pellicle layer and drying process was carried out using an oven at 37- 40 °C. Bacterial cellulose and its composites were ready to characterize.

Characterization of bacterial cellulose and its composites

Fourier transfrom infra-red (FTIR) spectra were recorded on a FTIR spectrophotometer (Shimadzu prestige 21, Japan) using the KBr disk technique. Morphological images of bacterial cellulose and its composites were investigated using a scanning electron microscope tool (SEM Jeol T300, USA).Tensile strength was characterized using a tensile tester (Universal Testing Machine UCT Series, Japan) equipped with a micrometer at a test speed of 10 mm/min. Thermogravimetric analyses were performed on a Differential Thermal Analyzer - Thermogravimatric Analyzer (Piris Diamond DTA/TG Perkin Elmer, Japan). About 15 mg of samples were heated at 30 to 400^0C with a heating rate of 10 ^{0}C/min under oxygen flow. X-ray diffractograms were recorded on an X-ray diffractometer (XRD Rigaku Mini Flex-6000 diffractometer, Japan) with radiation of Cu Kα. The scanning process was performed at speed of 4°/min in the range of 2 - 80°.

Antibacterial activity

Antibacterial activities of BC and its composites were investigated against *Staphylococcus aureus* by Wei *et al* method as described in [9]. *Staphylococcus aureus* isolates ATCC 25923 were rejuvenated by microbial inoculation on medium NA and incubated for 24 h at room temperature. *Staphylococcus aureus* ATCC 25923 was inoculated into NB medium in a bottle and incubated at 37^{o}C. After 24 h, optical density of *S. aureus* ATCC 25923 in NB media was measured. Since optical density (OD) of *S. aureus* ATCC 25923 in NB media was found 1, then 100 mL cultures of *S. aureus*was inoculated into a solid media of NA in petridish. Optical density 1 showed that the number of microbes in 1 mL of culture was about $1x10^8$. Microbial inoculation was conducted on a solid media in petri-dish using the spread plate method. Liquid culture (100 mL) in NB media was moved into petri-dish using a pipette tip aseptically. The BC and its composites were placed on the culture of microbes in the petri-dish, then incubated at 37°C. Inhibition zone of BC and its composites against *Staphylococcus aureus* ATCC 25923 was observed and measured by using calipers for 24 h. Clear zone around BC and its composites showed inhibition activity of BC toward microbial growth.

RESULTS AND DISCUSSIONS

Physical properties and functional groups

Table-1 shows that the mass of each cellulose and its composites is varied. However, the polymerization product of cellulose (BC and BC-G) exhibits similarities in color, transparency, smell, and texture. Bacterial cellulose-glycerol-chitosan (BC-G-Ch) composites is a light yellow color due to the presence of chitosan on its structure. Dry yield of BC, BC-G, and BC-G-Ch in Table 1 indicates that the bacterial cellulose is able to bind water up to 98% as reported by Lina *et al*[10].

Table-1. Physical characteristics of bacterial cellulose and its composites.

Parameter	BC	BC-G	BC-G-Ch
The wet mass (g)	132.25±11.74	140.86±20.25	122.40±25.68
The dry mass (g)	7.26±0.36	7.48±1.04	4.58±0.72
% Wet yield	66.13	70.43	61.20
% Dry yield	3.63	3.47	2.29
Transparency	Transparent	Transparent	Transparent
Color	White	White	Yellow
Odor	Odorless	Odorless	Acid
Texture	Flabby, watery	Flabby, watery	Rigid, dry

The dry mass of BC and BC-G composite was found much higher in comparison with BC-G-Ch composites. The BC and BC-G composites are able to absorb more water than the BC-G-Ch composite. The BC has mushy and watery texture. The adding chitosan on structure of BC will decrease its water absorption. These results correspond to the results reported by Sadikin *et al.*[11]. Chitosan is able to enter into the pores and inner surface of BC so that it blocks water to enter [12-14]. It is possible that hydrogen bonding between chitosan and the -OH group of BC might occur so that the water cannot bind -OH group.

Figure-1 shows that for the spectrum of BC-G-Ch, a wide band appears in the region 3400 cm^{-1} which indicates the presence of $–NH_2$ of chitosan and –OH of BC [15]. The band which appears around 1570 cm^{-1} for the three spectra indicates the presence of aromatic rings. The peak around 1558.48 cm^{-1} which appears on the spectrum of BC-G-Ch should correspond to amino groups of chitosan [7]. The typical stretching of C=O group of chitosan around 1635,64 cm^{-1} can be observed clearly in the spectrum of BC-G-Ch. The peaks of $-NH_2$ and C=O groups in this spectrum of the BC-G-Ch indicate that chitosan has been successfully attached on the structure of BC.

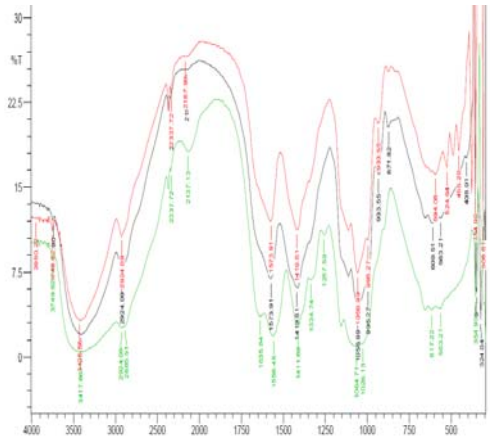


Figure-1. FTIR spectra of bacterial cellulose-glycerol/BC-G (upper), BC (medium), and bacterial cellulose-glycerol-chitosan/BC-G-Ch (lower).

Table-2 shows absorbance of –OH and –NH in the BC, BC-G and BC-G-Ch. The introduction of glycerol and chitosan in the structure of BC causes reducing the absorbance of the-OH group. The decreasing absorbance of -OH group may be caused by the formation of hydrogen bonding between cellulose and glycerol (chitosan). This will support the decreasing in percentage of elongation of bacterial cellulose as well as increasing thermal stability of bacterial cellulose-glycerol-chitosan. The presence of chitosan will broaden the band of -OH group on the spectrum of BCat around the3400cm^{-1}.

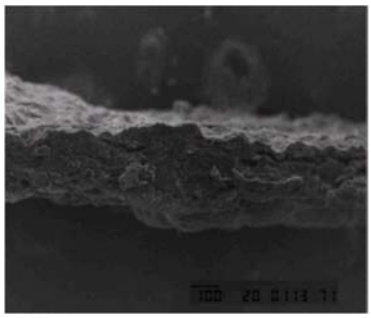
The broadening of this band indicates an overlapping between-OH and $-NH_2$groups due to the hydrogen bond [7]. Based on Figure-2, the presence ofthe bands at 635.64cm^{-1}and1566.20cm^{-1}associated with the stretching of C=O (Amide I) and bending of -NH (Amide II), respectively, suggests that the amide groups of chitinare not deacetylated perfectly [7].

Table-2. The absorbance of bacterial cellulose prepared from rice wastewater and its composites.

Wave number (cm^{-1})	Functional group	Absorbance		
		BC	BC-G	BC-G-Ch
3400	-OH	0.360	0.320	0.329
1635	-NH	-	-	0.116

Morphological images

Figure-2 shows morphological image ofBC, BC-G composite, and BC-G-Ch. Bacterial cellulose is composed by fibrilar organization where each fibril is comprised of a thousand of single linear glucan chain forming a net-like structure. Through SEM images it was noticed that a large deposition of glycerol over the BC surface covered the fibril. The cross section image showsbulk microfibrils denoting that glycerol can also penetrate through the surface pores of BC. However, the third image shows the difference of BC before and after introducing of chitosan. It is evident that chitosan was able to coat the entire surface of BC.



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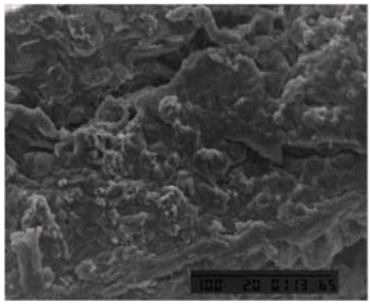

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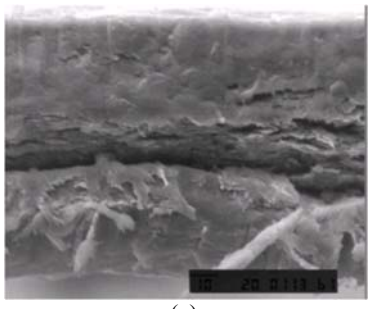


(c)

Figure-2. (a) Cross section photo of BC (100x), (b) surface photo of bacterial cellulose-glycerol composite (100x), (c).cross section photo of bacterial cellulose-glycerol-chitosan composite (100x).

Mechanical properties and crystallinity

As shown in Table-3 the tensile strength of BC and BC-G were found to be 22.48 MPa and 15.60 MPa, respectively. Decreasing in tensile strength was due to the presence of glycerol as a plasticizer agent that made material less rigid. Due to its lower rigidity, BC-G exhibits lower tensile strength and higher elongation in comparison to BC. Tensile strength of BC and BC-G were different. The percentage of elongation of BC-G was found 28.12% while in the BC it was only 22.18%. It isevident that the addition of glycerol as a plasticizer reduces the tensile strength but increases elongation of bacterial cellulose. Rechia*et al*. [16] studied about increasing the glycerol concentration in the film which reduced elasticity modulus and tensile strength, exhibiting a plasticizing effect.

However many researches such as Zhong and Xia [12] who tested the chemical and physical properties of a film that was added with glycerol as plasticizer agent showed that this can cause a decrease in tensile strength. This decreasing was due to reduced intermolecular interactions between cellulose and glycerol.

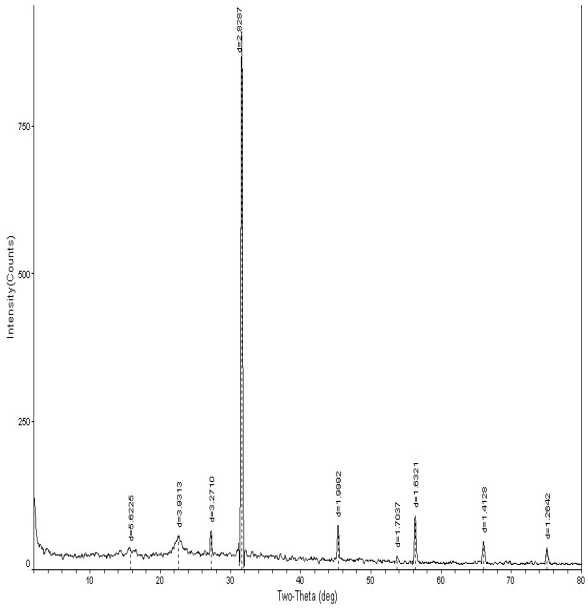
Table-3. Mechanical properties of bacterial cellulose and its composites.

Parameter	BC	BC-G	BC-G-Ch
Tensile strength (MPa)	22.48	15.60	17.01
Elongation (%)	22.18	28.12	8.01

The tensile strength of BC-G-Ch was found lower than that of BC but higher than that of BC-G (Table 3). The lower tensile strength of BC-G-Ch might be caused by chitosan which is amorphous, whereas the cellulose itself is a crystal. A strong structural material due to its higher crystallinity naturally is resilient to higher pressure than the irregular structure of materials and provided lots of space around it. The increasing of amorphous phase in the material which has a high crystallinity will reduce its strength at break of material [15]. The addition of chitosan decreased the elongation of composites significantly,from 28.12% for BC-G to 8.01% for BC-G-Ch. The use of cornstarch films can cause intermolecular bonds, forming hydrogen bonding. This bond increased tensile strength but decreased elongation [16].

The existence of rigid structure in the polymer chain will make it difficult to move when it is pulled so that elongation will decline [12]. A decline in the crystallinity of BC-G-Ch in comparison with BC was observed. A decrease in the crystallinity showed the addition of amorphous phase in the structure of BC. Chitosan was able to reduce the crystallinity of BC due its amorphous nature. The BC has a high crystallinity, which relatedto high mechanical properties of BC [3, 13]. Zhijiang[13] studied that the addition of chitosanon BC with the concentration rangefrom12to45 wt % decreased its tensile strength from 130MPa to 54MPa while the percentage of elongation dropped from 12% to 6.8%.

Figure-3 shows the XRD patterns of BC, BC-G, and BC-G-Ch. Diffraction peak at 15^0 is associated with the phases of cellulose 1α (1001α, 1101β, and 0101β), nevertheless peak at 22.5^0 reveals the phases planes of cellulose 1β (2001β)[2]. The BC shows diffraction peaks at above 22.5^0. For X-Ray diffractogram of BC-G and BC-G-Ch at above 30^0, the broadening peak corresponding to amorphous phase was observed. It indicates that the addition of glycerol and chitosan can change crystalline region in BC.



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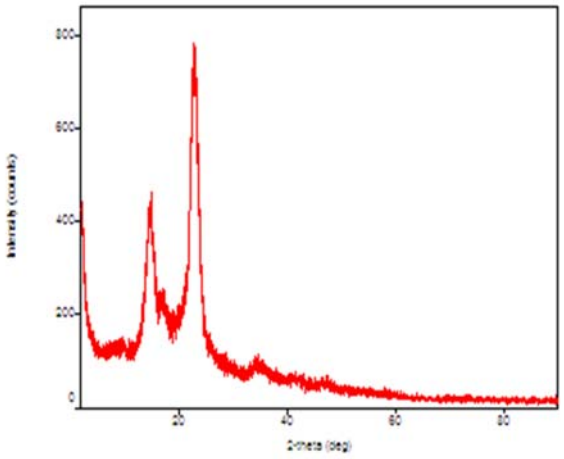

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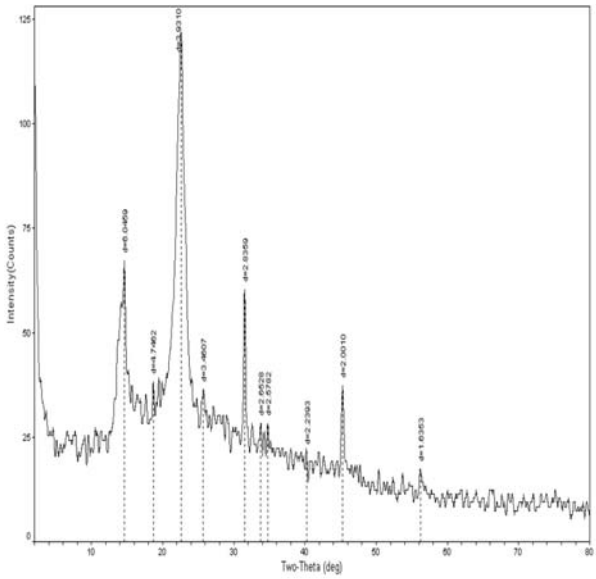

(c)

Figure-3. The X-Ray diffractogram of (a) BC; (b) BC-G; and (c) BC-G-Ch.

The presence of glycerol and chitosan caused a decreasing in the degree of crystallinity of BC. The degree of crystallinity of BC, BC-G, and BC-G-Ch was calculated and found to be 73.65%, 47%, and 50.15% respectively. Chitosan and glycerol are amorphous materials, whereas cellulose is a crystalline. The existence of the amorphous nature in BC caused deterioration of its crystallinity. The degree of crystallinity affected the mechanical properties of BC [16]. The decreasing degree of crystallinity can decrease tensile strength. The BC-G-Chexhibits higher tensile strength than the BC-G, due to its higher degree of crystallinity. Besides, the addition of chitosan will increase the tensile strength caused by the formation of hydrogen bonding between cellulose and chitosan so that the rigidity of the composite increased.

Thermal properties

Thermogram in Figure-4 shows endothermic peak as the release of water molecules on heating [2]. BC-G-Ch released water molecule atelevated temperature in comparison to BC and BC-G because the cellulose pores was blocked with chitosan, so that water molecules was trapped inside of cellulose structure. Glycerol and chitosan led to an increase in the amount of water molecules, consequently the endothermic peak of BC-G-Ch and BC-G occurred at higher temperatures than BC.

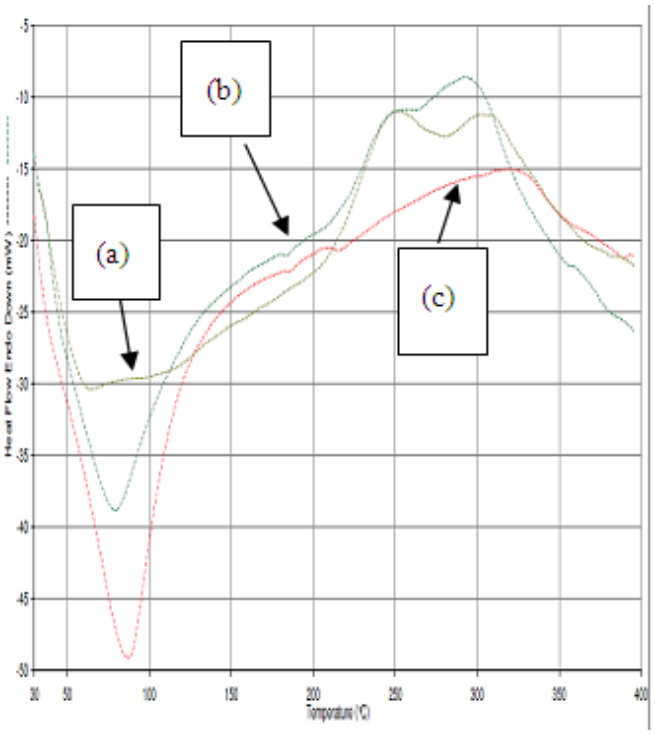

Figure-4. The DTA thermogram of BC (a); BC-G (b); and BC-G-Ch (c).

The peaks profiles suggest that a crystallization and/or phase transition preceded a thermal degradation. Crystallization and transition of glycerol and chitosan were an exothermic process continued thermal degradation of cellulose. Fragmentation process of carbonyl and carboxylic bonds in anhydrous glucoses units giving carbon or carbon monoxide is partial pyrolisis, and it can be observed as a broad exothermic peak [2]. Glass transition of BC was observed at 270 ^{0}C and crystallization of its was observed at 330^{0}C [2]. The BC and its composites were degradated at above 400^{0}C.

As shown in Figure-5, two significant losses of weight were observed at the temperature range of 37-100^{0}C and 200-350^{0}C. The first loss might be associated with dehydration of membrane. Water bound through hydrogen bonding and physically adsorbed can be detached at lower temperature of the first stage. The second loss was due to thermal degradation, and the BC-G exhibits the highest thermal stability. The first mass loss, going from 30 to 100^{0}C for membrane dehydration.

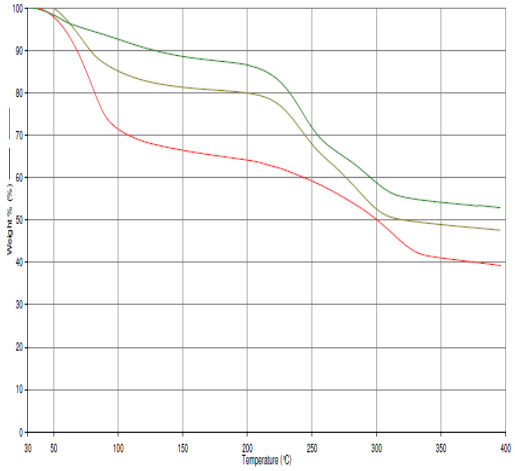

Figure-5. The TGA thermogram of BC-G (upper), BC (medium), and BC-G-Ch (lower).

Based on Figure-5, BC-G exhibits the lowest loss in mass in comparison to the other two. The BC-G-Ch composite indicates the highest loss in mass in comparison to the other two. The lowering thermal stability of BC-G-Ch in comparison to BC might be due to differences in the polymerization of the cellulose. Polymerization of cellulose without glycerol and chitosan forms linear polymers whereas when to the cellulose polymer is added the glycerol and chitosan it produces branching polymers. Chemical bonds in the linear polymer β-1,4-glycosidic are covalent, while in the branching polymer, hydrogen bonds are present. The covalent bond is stronger than the

hydrogen bonds so that the linear polymer is more difficult to decompose than the branching polymers [17]. Another possibility, because glycerol and chitosan is evaporated of polymers. It is also possible that the branching polymer is easily evaporated on the second stage. Composite of glycerol-polymer will evaporate at 200ºC temperature [14]. The BC-G exhibits higher thermal stability in comparison to the BC. This can be caused by the presence of hydrogen bonding between -OH group in celluose chain and –OH group in glycerol chain.

Antibacterial activity

Table-4 shows that the diameter of inhibition zone of BC was found the lowest in its composites. The presence of glycerol and chitosan on the structure of BC can increase its antibacterial activity. Chitosan can increase the permeability of the outer and inner membrane which can eventually damage the cell membranes of bacteria and followed by releasing of bacterial cell contents. This damage was due to the electrostatic interaction between $-NH_3^+$ groups of chitosan and phosphoryl group in the phospholipid bilayer in bacterial cell membranes [18]. Chitosan can interact with teichoic acid and forms an impermeable layer that prevents the entry of important molecules for the organism.

Table-4. Antibacterial activity of bacterial cellulose and its composites against *Staphylococcus aureus.*

Parameter	BC	BC-G	BC-G-Ch
Average Diameter of Inhibition Zone (mm)	0.096	11.600	11.800

The cell wall structure of the *Staphylococcus aureus*is thick, which is useful for protection from antibacterial agents such as antibiotics, toxins, chemicals and degradative enzymes. Peptidoglycan of *Staphylococcus aureus* is composed of teichoic acid, a polyalcohol which is linked with other sugars by phosphodiester bonds. Teichoic acid contains -COOH and phosphate groups, thus providing a negative charge on the surface of bacterial cells. Antibacterial activity of BC-G is caused by a covalent interaction between -OH groups in glycerol and COOH or -COO- group in the membranes of bacterial cell. It was confirmed by previous authors [19, 20] that glycerol with higher concentrationsexhibits antibacterial activity and can function as a good storage media for the cadaver allograft skin. Glycerol is safe to use in conserving the cadaver skin for several weeks at room temperature due to its antibacterial activity [19, 20].This study showed that all samples have antimicrobial activity against *Staphylococcus aureus*. Inhibition zone which occurs in cellulose is consistent with coordinate covalent bond, wherein the reducing of cellulose will react with a positive charge existing in the cell wall of the bacteria *Staphylococcus aureus*.

CONCLUSIONS

The addition of chitosan during preparation steps affected the physical properties of bacterial cellulose, the intensity of the functional groups, mechanical properties, the thermal stability, and its antibacterial activity. The tensile strength and crystallinity of bacterial celluloses decreased after the addition of chitosan. However, the addition of chitosan can increase intensity of functional group and antibacterial activity. The addition of glycerol can decrease the intensity of the functional groups, tensile strength, and crystallinity of composites but it can increase thermal stability, elongation, and antibacterial activity of bacterial cellulose.

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