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e-mail: eli_rohaeti@uny.ac.id; rohaetieli@yahoo.com
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Department of Chemistry Education
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| | |
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| No. | : A162264 |
| Title | : Bacterial Cellulose from Rice Waste Water with Addition of Glycerol and Chitosan |
| Authors | : Eli Rohaeti, Endang Widjajanti Laksono FX & Anna Rakhmawati |
| Adress | : Department of Chemistry Education, Faculty of Mathematics and Natural Sciences, Yogyakarta State University, Yogyakarta Indonesia Jl. Colombo No. 1 Yogyakarta 55281 |

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|--|--|----------|
| Cellulose is the most abundant natural biopolymer on the earth. It is naturally synthesized by plants, algae, and also some species of bacteria [1]. | Cellulose is a natural polymer material with a very high abundance, which is synthesized naturally by many plants, algae, and also an assortment of bacteria | 26 to 27 |
| trees and cotton | plants | 31 |
| In the culture medium, a pure cellulose network free of lignin and hemicellulose are obtained as highly hydrated pellicles made up of a random assembly of ribbon shaped fibers with size less than 100 nm wide. These fibers are composed of a bundle of finer micro fibrils of nano metric size [1, 2]. | A network of pure cellulose is free lignin and hemicellulose 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]. | 31 to 33 |
| The nano metric structure with unique properties have been applied in many commercial products including tires, headphone membranes, special papers, and textiles [2] and also in medical applications including temporary skin substitution [2, 5]. | Commercial product of cellulose can be found as an ingredient for making tires, membranes of headphones, special paper, textile [2], and biomedical applications as a temporary skin substitute, derived from nanometer-sized cellulose with unique properties [2, 5]. | 34 to 37 |
| Unlike cellulose from plants, (BC) is chemically pure, free of lignin and hemicelluloses. | The purity of bacterial celluloses is higher than cellulose of plants, they do not contain lignin and hemicellulose. | 38 to 39 |
| The BC does not require further process to remove unwanted polymers and contaminants | BC is obtained through a simple process, no further purification process to remove contaminants and unwanted polymer. | 39 to 40 |
| There are 2 common crystalline forms of cellulose, designated as cellulose I and cellulose II. | Cellulose I and cellulose II are two crystalline forms of cellulose. | 42 to 43 |
| Cellulose I is synthesized by the majority of plants and A. xylinum in static culture. | A. xylinum in static culture and most of the plants produced cellulose I. | 43 to 44 |
| The parallel structure of β -1,4-glucan chains are uniaxial. Whereas β -1,4-glucan chains of cellulose II are arranged in a random manner, antiparallel and linked with a larger number of hydrogen bonds that results in higher thermodynamic stability [1, 4]. | Cellulose I and cellulose II are composed of β -1,4-glucan chains with different structure. Cellulose I has parallel structure, whereas cellulose II has random and antiparallel structure, and also has hydrogen bonds, so that the cellulose II has a higher thermodynamic stability than cellulose I [1, 4]. | 42 to 47 |
| The water uptake of BC depends on the crystalline state of cellulose and the amount of grafted acrylic counterpart on its structure [2]. Cellulose phosphate membranes were also studied concerning their biomedical applications [2]. Phosphorylated cellulose seems to be interesting material for bone regeneration and osteointegration [2]. | 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 osteointegration has been studied from phosphorylated cellulose. [2] | 49 to 53 |

| | | |
|---|---|------------|
| The BC has unique structure and mechanical properties in comparison with higher plant cellulose. | The BC has mechanical properties and specific structure in comparison with cellulose of plants. | 54 to 55 |
| The BC fibers have a high aspect ratio with a diameter of 20-100 nm. As a result, BC has a very high surface area per unit mass. This property combined with its highly hydrophilic nature results high liquid loading capacity material. Moreover, its biocompatibility makes an prospective candidate for a wide range of applications in different fields, especially those related to biomedical and biotechnology [5]. | 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 which has a large surface area with highly hydrophilic properties, the resulting material can be applied in various fields with a very wide spectrum, particularly for applications in the fields of biomedical and biotechnology [5]. | 55 to 59 |
| However, in most practical applications, BC has low quality and it is costly for industry. For economical mass production, it is essential to design an effective and efficient culture aeration and agitation process [6]. | 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. | 59 to 62 |
| chitosan is effective in inhibiting the growth of bacteria [7, 8, 11]. | a bacterial growth can be inhibited effectively by chitosan [7, 8, 11] | 74 to 75 |
| Wei et al.[9] stated that Staphylococcus aureus is one of gram-positive bacteria generally found on contaminated wounds. | Wei <i>et al.</i> [9] stated that generally in a contaminated wounds are often found gram-positive bacteria in the form of <i>Staphylococcus aureus</i> | 81 to 82 |
| was examined by using a scanning electron microscope (SEM Jeol | was investigated by a scanning electron microscope tool (SEM Jeol) | 98 to 99 |
| Mechanical properties were determined by using a tensile tester (Universal Testing Machine | Mechanical properties were measured by a Universal Testing Machine (Tensile Tester UCT Series, Japan) | 99 to 101 |
| pH of solution was set to 3-4 by using glacial acetic acid | pH of solution is maintained at pH 3-4 by the addition of acetic acid | 111 to 112 |
| dried in an oven with at 37- 40°C | drying process is carried out using an oven at a temperature 37- 40 °C | 117 to 118 |
| were examined by using a scanning electron microscope | were investigated using a scanning electron microscope tool | 123 to 124 |
| Mechanical properties were examined with a Universal Testing Machine | Tensile strength was characterized using a tensile tester | 124 |
| from room temperature to 400 °C at a rate of 10 °C/min under | from 30 °C until 400 °C at a heating rate of 10 °C/min with oxygen flow | 128 to 129 |
| with Cu Kα radiation at 40 kV and 40 mA | at 40 kV and 40 mA with radiation of Cu Kα . | 129 to 130 |
| Scanning were performed on a scan | The scanning process is performed at speed | 130 |
| Physical properties of BC, BC-G, and BC-G-Ch | Physical characteristic of bacterial cellulose and its composites | 159 |
| presence of C = O stretching (Amide I) and-NH bending (Amide II) | stretching of C = O (Amide I) and bending of -NH (Amide II) | 194 to 195 |
| SEM image of bacterial cellulose (cross section, 100x), (b). SEM image of bacterial cellulose-glycerol composite (surface, 100x), (c). SEM image of bacterial cellulose-glycerol-chitosan composite (cross section, 100x) | Cross section photo of BC (100x) Surface photo Cross section photo of | 213 to 214 |
| a contribution of an exothermic process followed by an endothermic process. | a crystallization and/or phase transition preceded a thermal degradation. | 265 to 266 |
| Crystallization and/or phase transition of glycerol and chitosan lead to the exothermic event followed the thermal degradation of the cellulose | Crystallization and transition of glycerol and chitosan were an exothermic process continued thermal degradation of cellulose. | 268 to 269 |

| | | |
|--|--|------------|
| | | |
| The broad exothermic peak can be attributed to the partial pyrolysis due to fragmentation of carbonyl and carboxylic bonds from anhydrous glucoses units giving carbon or monoxide carbon [2] | Fragmentation process of carbonyl and carboxylic bonds from anhydrous glucoses units giving carbon or monoxide carbon is partial pyrolysis, can be observed as a broad exothermic peak [2]. | 269 to 271 |
| Glass transition and crystallization were observed in BC at 270 and 330^oC, respectively | Glass transition of BC was observed at 270 ^o C and crystallization of its was observed at 330 ^o C | 273 to 274 |
| Two significant mass losses may be observed from room temperature to 100^oC and from 100 to | From 37 until 100 ^o C and from 100 until 400 ^o C observed two significant weight losses | 276 to 277 |
| due to membrane dehydration. Physically adsorbed and hydrogen bond linked water molecules can be lost at the first stage. The second mass loss was due to | Caused by dehydration of membrane. Water bound through hydrogen bonding and physically adsorbed can be detached at the first stage. The second weight loss | 278 to 279 |
| Diffraction peaks at 15 and 22.50 were assigned to the cellulose 1α and 1β phases (100$1\alpha$, 110$1\beta$, and 010$1\beta$ planes at 15^o and 110$1\alpha$, and 200$1\beta$ at 22.50) [2] | Diffraction peak at 15 ^o showed the phases of cellulose 1 α (100 1α , 110 1β , and 010 1β), nevertheless peak at 22.5 ^o revealed the phases planes of cellulose 1 β (200 1β) | 309 to 311 |
| of BC (a); BC-G (b); and BC-G-Ch (c) | of (a) the BC; (b) the BC-G; and (c) the BC-G-Ch | 321 |
| strength due to the formation of hydrogen bonding between cellulose with chitosan that increased its rigidity. | caused by hydrogen bonding between cellulose with chitosan so that the rigidity of the composite increased | 331 to 332 |
| glycerol in higher concentrations was an appropriate storage medium for allograft cadaver skin and has been attributed an antimicrobial effect. Glycerol had antibacterial activity and microbial safety of glycerol-preserved skin can be increased by preserving skin allografts for some weeks at room temperature [20, 21]. | glycerol with higher concentrations has 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 [20, 21]. | 355 to 358 |
| The results of this study showed that all the samples | This study showed that all samples | 359 |
| of bacterial cellulose. The mechanical properties and crystallinity of BCs | its. The tensile strength and crystallinity of bacterial celluloses | 367 to 368 |

Bacterial Cellulose from Rice Waste Water with Addition of Glycerol and Chitosan

Eli Rohaeti¹, Endang Widjajanti Laksono FX¹ & Anna Rakhmawati²

¹ Department of Chemistry Education, Faculty of Mathematics and Natural Sciences,
Yogyakarta State University, Yogyakarta Indonesia
Jl. Colombo No. 1 Yogyakarta 55281

² Department of Biology Education, Faculty of Mathematics and Natural Sciences,
Yogyakarta State University, Yogyakarta Indonesia
Jl. Colombo No. 1 Yogyakarta 55281

Email: eli_rohaeti@uny.ac.id; rohaetieli@yahoo.com

Abstract. Bacteria cellulose (BC) and its composites were synthesized from rice waste water (RWW) with 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 FTIR, XRD, and TGA-DTA confirmed the presence of glycerol and chitosan in BC composites. The antibacterial activity of BC and its composites were evaluated against *Staphylococcus aureus* ATCC 25923 by clear zone method. The experimental data showed that BC-G-Ch have a higher antibacterial activity in comparison with BC and BC-G.

Keywords: *Acetobacter xylinum*, antibacterial activity, bacterial cellulose, chitosan, glycerol

1 Introduction

Cellulose is a natural polymer material with a very high abundance, which is synthesized 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 obtained in a culture medium as a pellicle composed of fibers with a size less

33 than 100 nm. These cellulose fibers are composed of fine threads nanoscale. [1,
34 2]. Commercial product of cellulose can be found as an ingredient for making
35 tires, membranes of headphones, special paper, textile [2], and biomedical
36 applications as a temporary skin substitute [2, 5], derived from nanometer-sized
37 cellulose with unique properties.

38 The purity of bacterial celluloses is higher than cellulose of plants, they do not
39 contain lignin and hemicellulose. BC is obtained through a simple process, no
40 further purification process to remove contaminants and unwanted polymer,
41 therefore, retains a greater degree of polymerization [3]. The BC presences in
42 high christalinity. Cellulose I and cellulose II are two crystalline forms of
43 cellulose. *A. xylinum* in static culture and most of the plants produced cellulose
44 I. Cellulose I and cellulose II are composed of β -1,4-glucan chains with
45 different structure. Cellulose I has parallel structure, whereas cellulose II has
46 random and antiparallel structure, and also has hydrogen bonds, so that the
47 cellulose II has a higher thermodynamic stability than cellulose I. [1, 4]. The BC
48 is also used as dietary fiber and as a binding or thickening agent [1].
49 Crystallinity and the amount of acrylic that can be grafted into the cellulose
50 structure will affect the ability of BC to absorb water. Biomedical material has
51 been studied from a cellulose phosphate membrane. Similarly, bone
52 regeneration applications and osteointegration has been studied from
53 phosphorylated cellulose [2].

54 The BC has unique structure and mechanical properties in comparison with
55 higher plant cellulose. A surface area per unit mass of the BC fiber is high, as
56 a result of its diameter of 20-100 nm. The combined properties of cellulose
57 which has a large surface area with highly hydrophilic properties, the resulting
58 material can be applied in various fields with a very wide spectrum, particularly
59 for applications in the fields of biomedical and biotechnology [5]. It is

60 important to design an efficient production of BC by aeration and agitation
61 method, so it can be produced effectively with good quality and economical for
62 industrial-scale production [6]

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

71 Medical applications of BC primarily aims to prevent infection by pathogenic
72 microorganisms. Addition of chitosan on BC is aimed to provide antimicrobial
73 properties to cellulose. Chitosan is a glucosamine biopolymer produced from
74 deacetylation of chitin. Recent studies claim that a bacterial growth can be
75 inhibited effectively by chitosan [7, 8, 11]. Chitosan has a positively charged
76 amine groups that will bind to the negatively charged groups on the surface of
77 microorganisms [8]. Institute of Chemical Fibers (ICWH) Poland has been
78 synthesized BC composite by introducing chitosan on BC [8].

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

86 2 Experimental Section

87 2.1 Materials

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

95 2.2 Instrumentation

96 Organic functional groups of BC and its composites were analyzed by using a
97 Fourier Transform Infrared (FT-IR) spectrophotometer (Shimadzu prestige
98 21, Japan). Surface morphological was examined by using a scanning electron
99 microscope tool (SEM Jeol T300, USA). Mechanical properties were
100 determined by using a tensile tester (Universal Testing Machine UCT Series,
101 Japan). Thermal properties were examined by using a Differential Thermal
102 Analyzer and Thermogravimetric Analyzer (Piris Diamond DTA/TG Perkin
103 Elmer, Japan). X-Ray Diffraction (XRD) pattern was recorded by using a XRD
104 Rigaku Mini Flex-6000 diffractometer, Japan.

105 2.3 Procedure

106 2.3.1 Preparation of Bacterial Cellulose and Its Composites

107 Rice wastewater was prepared by washing a kilogram of rice by a liter of
108 aquadest. The mixture was filtered and the filtrate was collected in a container.
109 The BC-G-Ch was prepared as follow: Twenty grams of sucrose, 1.0 g of urea,

110 and 1.0 g glycerol were mixed with 200 mL RWW. The mixture was poured
111 into Erlenmeyer and stirred magnetically until dissolved and the pH of solution
112 was set to 3-4 by using glacial acetic acid. Subsequently, the mixture was
113 poured into a tray and cooled to room temperature. *Acetobacter xylinum* (40
114 mL) was introduced into the solution and fermented for 7-14 days at room
115 temperature. The formed pellicle layer was washed several times with tap water,
116 distilled water and hot water, respectively. Then 2 wt.% chitosan solution with
117 deacetylation degree of 73.78% was poured onto the pellicle layer and drying
118 process is carried out using an oven at a temperature 37- 40 °C. Bacterial
119 cellulose and its composites were ready to be characterized.

120 2.3.2 Characterization of Bacterial Cellulose and Its Composites

121 Fourier transform infra-red (FTIR) spectra was measured on a FTIR using the
122 KBr disk technique. Morphological images of bacterial cellulose and its
123 composites were investigated using a scanning electron microscope tool.
124 Tensile strength was characterized using a tensile tester, which is equipped
125 with a micrometer Mitutoyo, Japan at a test speed of 10 mm/min.
126 Thermogravimetric analyses were performed on a Differential Thermal
127 Analysis – Thermogravimetric Analysis (DTA - TGA, Japan). Sample (15 mg)
128 were heated from 30 °C until 400 °C at a heating rate of 10 °C/min with oxygen
129 flow. X-ray diffractograms were recorded on a X-ray diffractogram at 40 kV
130 and 40 mA with radiation of Cu K α . The scanning process is performed at
131 speed of 4°/min in the range of 2 - 80°.

132 2.3.3 Antibacterial activity

133 Antibacterial activities of BC and its composites were investigated against
134 *Staphylococcus aureus* by Wei *et al* method as described [9]. *Staphylococcus*
135 *aureus* isolates ATCC 25923 were rejuvenated by microbial inoculation on

136 medium NA and incubated for 24 h at room temperature. *Staphylococcus*
 137 *aureus* ATCC 25923 was inoculated into NB medium in bottle and incubated at
 138 37°C. After 24 h, optical density of *S. aureus* ATCC 25923 in NB media was
 139 measured. If optical density (OD) of *S. aureus* ATCC 25923 in NB media has
 140 shown 1, then 100 mL cultures of *S. aureus* was inoculated into a solid media of
 141 NA in petridish. Optical density 1 showed that the number of microbes in 1 mL
 142 of culture is as much as 1×10^8 . Microbial inoculation was conducted on a solid
 143 media in petridish using the spread plate method. Liquid culture (100 mL) in
 144 NB media was moved into petridish using a pipette tip aseptically. The BC and
 145 its composites were placed on the culture of microbes in the petridish, then
 146 incubated at 37°C. Inhibition zone of BC and its composites against
 147 *Staphylococcus aureus* ATCC 25923 was observed and measured by using
 148 calipers for 24 h. Clear zone around BC and its composites showed inhibition
 149 activity of BC toward microbial growth.

150 3. Results and Discussion

151 3.1 Physical Properties

152 Table 1 showed that the mass of each cellulose and its composites was varied.
 153 However, the polymerization product of cellulose (BC and BC-G) had
 154 similarities on color, transparency, smell, and texture. Bacterial cellulose-
 155 glycerol-chitosan (BC-G-Ch) composites had a light yellow color due to the
 156 presence of chitosan on its structure. Dry yield of BC, BC-G, and BC-G-Ch in
 157 Table 1 indicated that the bacterial cellulose was able to bind water up to 98%
 158 as reported by Lina *et al* [17].

159 **Table 1** Physical characteristic 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 |

160

161 The dry mass of BC and BC-G composite was much higher in comparison with
 162 BC-G-Ch composites. The BC and BC-G composites were able to absorb more
 163 water than the BC-G-Ch composite. The BC has mushy and watery texture. The
 164 adding chitosan on structure of BC will decrease its water absorption. These
 165 results correspond to the results reported by Sadikin *et al.* [18]. Chitosan was
 166 able to enter into the pores and inner surface of BC so that blocks water to enter.
 167 [10-12]. Another possibility, there is hydrogen bonding between chitosan and
 168 the -OH group of BC so that the water can not bind -OH group.

169 **3.2 Functional Groups**

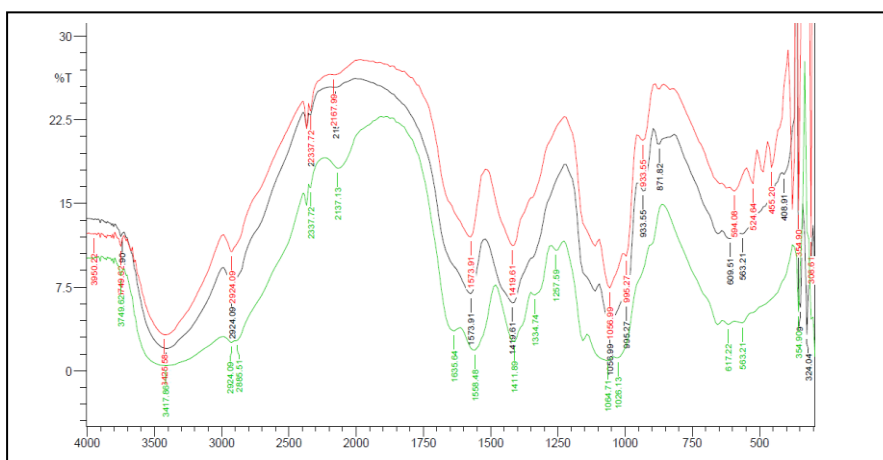
170 Figure 1 showed that for spectra of BC-G-Ch, a wide peak appeared in the
 171 region 3400 cm^{-1} which indicates the presence of -NH_2 of chitosan and -OH of
 172 BC [13]. Three spectra appeared around 1570 cm^{-1} indicating the presence of
 173 aromatic rings. Peak around 1558.48 cm^{-1} , appeared on spectra of BC-G-Ch
 174 which corresponded to amino groups of chitosan [7]. The characteristic peak of
 175 $\text{C}=\text{O}$ group of chitosan around 1635 cm^{-1} can be observed in the spectra of BC-
 176 G-Ch. The peaks of -NH_2 and $\text{C}=\text{O}$ groups in spectra of the BC-G-Ch indicate
 177 that chitosan has successfully attached on the structure of BC.

178

179

180

181



182 **Figure 1** FTIR spectrum of bacterial cellulose-glycerol/BC-G (upper), BC (medium),
 183 and bacterial cellulose-glycerol-chitosan/BC-G-Ch (lower)

184 Table 2 showed absorbance of –OH and –NH in The BC, BC-G and BC-G-Ch.
 185 Presence of glycerol and chitosan in the structure of BC decreased the
 186 absorbance of the-OH group. The decreasing absorbance of –OH group was
 187 caused by hydrogen bonding between cellulose and glycerol (chitosan). This
 188 will support the decreasing in percent elongation of bacterial cellulose when
 189 added with chitosan as well as increasing thermal stability of bacterial cellulose-
 190 glycerol-chitosan. The presence of chitosan will broaden peak-OH group on the
 191 spectra of BC around the 3400 cm⁻¹.

192 The broadening of this peak indicated an overlapping between -OH group and -
 193 NH₂ groups due to hydrogen bond [7]. Based on Figure 2, the visible presence
 194 of 635.64 cm⁻¹ and 1566.20 cm⁻¹ indicating the presence of **stretching of C = O**
 195 **(Amide I) and bending of -NH (Amide II)**, showed amide groups of chitin were
 196 not deacetylated perfectly [7].

197 **Table 2** The absorbance of bacterial cellulose 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 |

198

199 3.3 Morphological images of BC and its composites

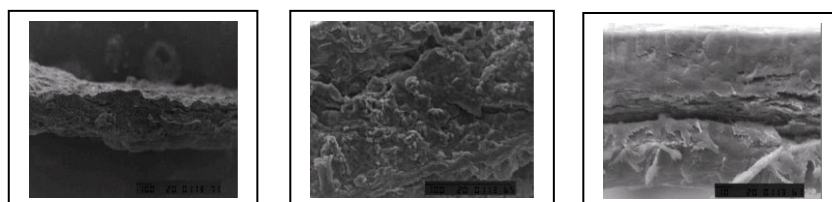
200 Figure 2 showed morphological image of BC, BC-G composite, and BC-G-Ch.
 201 Bacterial cellulose was composed by fibrillar organization where each fibril was
 202 comprised of a thousand of single linear glucan chain forming a net-like
 203 structure. Through SEM images it was noticed that a large deposition of
 204 glycerol over the BC surface covered the fibril. The cross section image showed
 205 bulk microfibrils denoting that glycerol can also penetrate through the surface

206 pores of BC. However, the third image showed the difference of BC before and
 207 after introducing of chitosan. It is proved that chitosan was able to coat the
 208 entire surface of BC.

209

210

211



212

(a)

(b)

(c)

213 **Figure 2** (a). Cross section photo of BC (100x), (b) Surface photo of bacterial
 214 cellulose-glycerol composite (100x), (c). Cross section photo of bacterial cellulose-
 215 glycerol-chitosan composite (500x)
 216

217 3.4 Mechanical Properties

218 Table 3 showed that tensile strength of BC and BC-G were 22.48 MPa and
 219 15.60 MPa, respectively. Decreasing in tensile strength was due to the presence
 220 of glycerol as a plasticizer agent that made material less rigid. Due to its lower
 221 rigidity, BC-G has lower tensile strength and higher elongation in comparison
 222 with BC. Tensile strength of BC and BC-G were different. Percentage of
 223 elongation of BC-G was 28.12 % while in the BC only 22.18% . It was proved
 224 that the addition of glycerol as a plasticizer decreased tensile strength but
 225 increased elongation of bacterial cellulose. Rechia *et al.* [15] studied about the
 226 increasing the glycerol concentration in the film reduced elasticity modulus and
 227 tensile strength, exhibiting a plasticizing effect.

228 These experimental results were consistent with the theory. However many
 229 researches such as Zhong and Xia [10] who tested the chemical and physical
 230 properties of a film that was added with glycerol as plasticizer agent showed

231 that the addition of glycerol can cause a decrease in tensile strength. This
 232 decreasing was due to reduced intermolecular interactions between cellulose
 233 and glycerol.

234 **Table 3** Mechanical properties of bacterial cellulose and its composites

| Parameter | BC | BC-G | BC-G-Ch |
|-------------------------------|-----------|-------------|----------------|
| <i>Tensile strength (MPa)</i> | 22.48 | 15.60 | 17.01 |
| <i>Elongation (%)</i> | 22.18 | 28.12 | 8.01 |

235

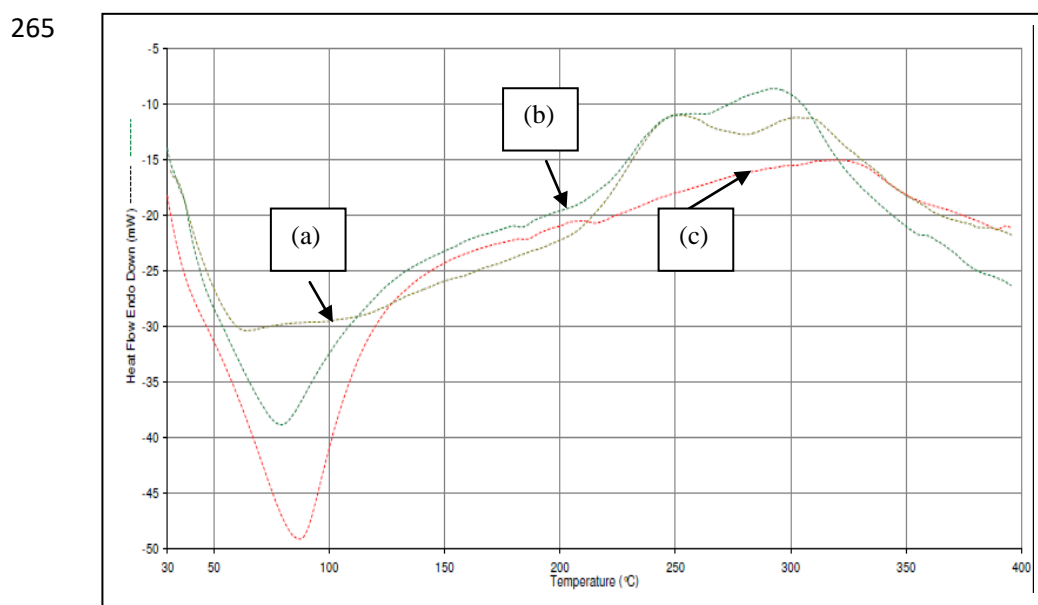
236 Tensile strength of BC-G-Ch is lower than BC but higher than BC-G. The
 237 decreasing of tensile strength of BC-G-Ch is caused by chitosan which is
 238 amorphous, whereas the cellulose itself is a crystal. A strong structural material
 239 due to its higher crystallinity naturally resilient to higher pressure, than the
 240 irregular structure of materials and provided lots of space around it. The
 241 increasing of amorphous phase in the material which has a high crystallinity
 242 will reduce its strength at break of material [14]. The adding of the chitosan
 243 decreased elongation of composites significantly. Elongation of BC-G were
 244 initially 28.12% and had decreased to 8.01% after the adding of chitosan. The
 245 use of cornstarch films can cause intermolecular bonds, formed hydrogen
 246 bonding. This bond increased tensile strength but decreased elongation [15].

247 The existence of the rigid structure in the polymer chain will make it difficult to
 248 move when it is pulled so that elongation will decline [10]. A decline in the
 249 crystallinity of BC-G-Ch in comparison with BC was observed. A decrease in
 250 the crystallinity showed the addition of amorphous phase in the structure of BC.
 251 Chitosan was able to reduce the crystallinity of BC due its amorphous nature.
 252 The BC has a high crystallinity, which related with a high mechanical properties
 253 of BC [3, 11]. Zhijiang [11] studied that the addition of chitosan on BC with
 254 concentration range from 12 to 45 wt.%, decrease its tensile strength from 130

255 MPa to 54 MPa while the value of percent elongation dropped from 12% to
 256 6.8%.

257 3.5 Thermal Properties

258 Thermogram in Fig. 3 showed endothermic peak as the release of water
 259 molecules [2]. BC-G-Ch released water molecule in higher temperature in
 260 comparison with BC and BC-G because the cellulose pores was blocked with
 261 chitosan, so that water molecules was trapped inside of cellulose structure.
 262 Glycerol and chitosan led to an increase in the amount of water, consequently
 263 endothermic peak of BC-G-Ch and BC-G occurred at higher temperatures than
 264 BC.



266 **Figure 3** The DTA thermogram of BC (a); BC-G (b); and BC-G-Ch (c)

267
 268 The peaks profiles suggested that a crystallization and/or phase transition
 269 preceded a thermal degradation. Crystallization and transition of glycerol and
 270 chitosan were an exothermic process continued thermal degradation of

271 cellulose. Fragmentation process of carbonyl and carboxylic bonds from
 272 anhydrous glucoses units giving carbon or monoxide carbon is partial pyrolysis,
 273 can be observed as a broad exothermic peak [2]. Glass transition of BC was
 274 observed at 270 °C and crystallization of its was observed at 330°C [2]. The BC
 275 and its composites were degraded at above of 400°C.

276 From 37 until 100°C and from 100 until 400°C observed two significant weight
 277 losses (Fig 4). The first weight loss, occurred from 30 to 100°C caused by
 278 dehydration of membrane. Water bound through hydrogen bonding and
 279 physically adsorbed can be detached at the first stage. The second weight loss
 280 was due to thermal degradation. The first mass loss , going from 30 to 1000C
 281 for membrane dehydration . Physically adsorbed and related water molecules
 282 hydrogen bond could be lost in the first stage . The second mass loss is due to
 283 thermal degradation .The BC-G had the highest thermal stability.

284

285

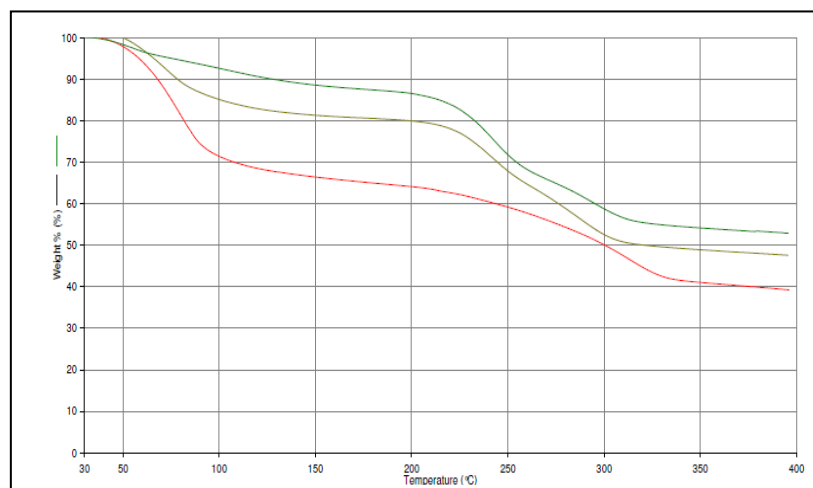
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292

Figure 4 The TGA thermogram of BC-G (upper), BC (medium), and BC-G-Ch (lower)

293

294

295

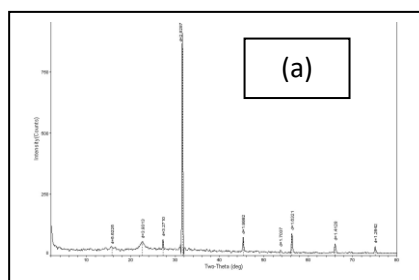
Based on Fig. 4, BC-G has the lowest mass loss in comparison with both of BC and BC-G-Ch. The BC-G-Ch composite has the highest mass loss in comparison with both of BC and BC-G. Decreasing in thermal stability of BC-

296 G-Ch in comparison with BC might be due to differences in the polymerization
 297 of the cellulose. Polymerization of cellulose without glycerol and chitosan form
 298 linear polymers whereas the cellulose polymer is added to the glycerol and
 299 chitosan producing branched polymers. Chemical bonds in the linear polymer β -
 300 1,4-glycosidic are covalent, while in the branched polymer are hydrogen bonds.
 301 The covalent bond is stronger than hydrogen bonds so that the linear polymer is
 302 more difficult to decompose than branched polymers [16]. Another possibility,
 303 because glycerol and chitosan is evaporated of polymers. Composite of
 304 glycerol-polymer will evaporated at 200°C temperature [12]. The BC-G has
 305 higher thermal stability in comparison with the BC. This can be caused by
 306 hydrogen bonding between -OH group in cellulose chain and -OH group in
 307 glycerol chain.

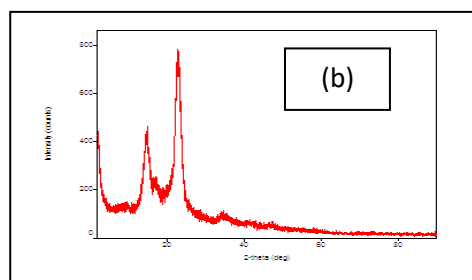
308 3.6 Crystallinity

309 Fig. 5 showed the XRD patterns of BC, BC-G, and BC-G-Ch. Diffraction peak
 310 at 15° showed the phases of cellulose 1α (1001α , 1101β , and 0101β),
 311 nevertheless peak at 22.5° revealed the phases planes of cellulose 1β (2001β)
 312 [2]. The BC showed diffraction peaks at above 22.5° . For X-Ray diffractogram
 313 of BC-G and BC-G-Ch at above of 30° , broaden peak corresponded to
 314 amorphous phase was observed. It indicates that addition of glycerol and
 315 chitosan can change crystalline region in BC.

316



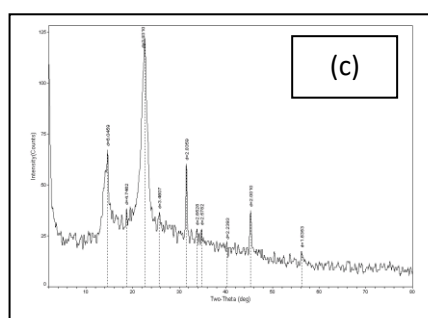
317



318

319

320



321 **Figure 5** The X-Ray diffractogram of (a) the BC; (b) the BC-G; and (c) the BC-G-Ch
 322 The presence of glycerol and chitosan caused a decreasing in the degree of
 323 crystallinity of BC. Based on the results of calculation of crystallinity degree, it
 324 showed that degree of crystallinity of BC, BC-G, BC-G-Ch was 73.65%, 47%
 325 and 50.15% respectively. Chitosan and glycerol is an amorphous material,
 326 whereas cellulose is a crystal. The existence of the amorphous nature in the BC
 327 caused deterioration of its crystallinity. The degree of crystallinity affected the
 328 mechanical properties of BC [15]. The decreasing degree of crystallinity can
 329 decrease tensile strength. The BC-G-Ch had higher tensile strength than the
 330 BC-G, due to its higher degree of crystallinity. Besides, the adding chitosan will
 331 increase the tensile strength caused by hydrogen bonding between cellulose
 332 with chitosan so that the rigidity of the composite increased.

333 3.7 Antibacterial Activity

334 Table 4 showed that inhibition zone diameter of BC was the lowest in
 335 comparison with its composites. The presence of glycerol and chitosan on the
 336 structure of BC can increase its antibacterial activity. Chitosan can increase the
 337 permeability of the outer and inner membrane which can eventually damage the
 338 cell membranes of bacteria and followed by releasing of bacterial cell contents.
 339 This damage due to the electrostatic interaction between $-NH_3^+$ groups of
 340 chitosan with phosphoryl group in the phospholipid bilayer in bacterial cell
 341 membranes [19]. Chitosan can interact with teichoic acid and formed an
 342 impermeable layer that prevented the entry of important molecules for the
 343 organism.

344 **Table 4** Antibacterial activity of bacterial cellulose and its composites against
 345 *Staphylococcus aureus*

| Parameter | BC | BC-G | BC-G-Ch |
|-----------|----|------|---------|
|-----------|----|------|---------|

| | | | |
|---|-------|--------|--------|
| <i>Average of Inhibition Zone Diameter (mm)</i> | 0.096 | 11.600 | 11.800 |
|---|-------|--------|--------|

346

347 The cell wall structure of the *Staphylococcus aureus* was thick, which is useful
 348 for protection from antibacterial agents such as antibiotics, toxins, chemicals
 349 and degradative enzymes. Peptidoglycan of *Staphylococcus aureus* is
 350 composed of teicoic acid, a polyalcohol which is linked with other sugars by
 351 phosphodiester bonds. Teicoic acid has -COOH group and phosphate group,
 352 thus providing a negative charge on the surface of bacterial cells. Antibacterial
 353 activity of BC-G caused by a covalent interaction between -OH groups in
 354 glycerol with COOH or -COO- group in bacterial cell membranes. It was
 355 confirmed by previous authors that glycerol with higher concentrations has
 356 antibacterial activity and can function as a good storage media for the cadaver
 357 allograft skin. Glycerol is safe to use in conserving the cadaver skin for several
 358 weeks at room temperature due to its antibacterial activity [20, 21].

359 This study showed that all samples have antimicrobial activity against
 360 *Staphylococcus aureus*. Inhibition zone which occurred in cellulose was
 361 consistent with theory of coordinate covalent, wherein the reducing of cellulose
 362 will react with a positive charge contained in the cell wall of the bacteria
 363 *Staphylococcus aureus*.

364 **4 Conclusion**

365 The adding of chitosan during preparation steps affected the physical properties
 366 of bacterial cellulose, the intensity of the functional groups, mechanical
 367 properties, the thermal stability, and its antibacterial activity. The tensile
 368 strength and crystallinity of bacterial celluloses decreased after adding of
 369 chitosan. However, the adding of chitosan can increase intensity of functional
 370 group and antibacterial activity. The adding of glycerol can decrease the

371 intensity of the functional groups, tensile strength, and crystallinity of
372 composites but can increase thermal stability, elongation, and antibacterial
373 activity of bacterial cellulose.

374 **5. Acknowledgements**

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

Eli Rohaeti¹, Endang Widjajanti Laksono FX¹ and Anna Rakhmawati²

¹Chemistry Education Department, Faculty of Mathematics and Natural Sciences, Yogyakarta State University, Yogyakarta, Indonesia

²Biology Education Department, Faculty of Mathematics and Natural Sciences, Yogyakarta State University, Yogyakarta, Indonesia

E-Mail: eli_rohaeti@uny.ac.id; rohaetieli@yahoo.com

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: *acetobacter xylinum*, antibacterial activity, bacterial cellulose, chitosan, glycerol.

INTRODUCTION

Cellulose is an abundant 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 can be 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 [2], and biomedical applications as a temporary skin substitute [2, 5] 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 crystallinity. 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 osteointegration have been studied from phosphorylated cellulose [2].

The BC exhibits unique 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 an infection by pathogenic microorganisms. Addition of chitosan on BC is aimed to provide antimicrobial properties to cellulose. Chitosan is a glucosamine biopolymer produced from deacetylation of chitin. Recent studies claimed that a bacterial growth can be inhibited effectively by chitosan [7, 8, 11]. Chitosan has a positively charged amine group that will bind to the negatively charged group on the surface of microorganisms [8]. Institute of Chemical Fibers (ICWH) Poland has synthesized BC composite by introducing chitosan on BC [8].



According to TheNational Nosocomial Infection Surveillance System(NNIs), *Staphylococcus aureus* is one of the mostcommonpathogensin 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 purchased as 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 aquadest. 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 into an Erlenmeyer and stirred magnetically 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. *Acetobacter xylinum* (40mL) was then introduced into the solution and fermented for 7-14 days at 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 transform 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 - Thermogravimetric Analyzer (Piris Diamond DTA/TG Perkin Elmer, Japan). About 15 mg of samples were heated at 30 to 400°C with a heating rate of 10 °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 K α . 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°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 1×10^8 . 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* [17].

**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.* [18]. Chitosan is able to enter into the pores and inner surface of BC so that it blocks water to enter [10-12]. 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 $-\text{NH}_2$ of chitosan and $-\text{OH}$ of BC [13]. 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 $\text{C}=\text{O}$ group of chitosan around 1635.64 cm^{-1} can be observed clearly in the spectrum of BC-G-Ch. The peaks of $-\text{NH}_2$ and $\text{C}=\text{O}$ groups in this spectrum of the BC-G-Ch indicate that chitosan has been successfully attached on the structure of BC.

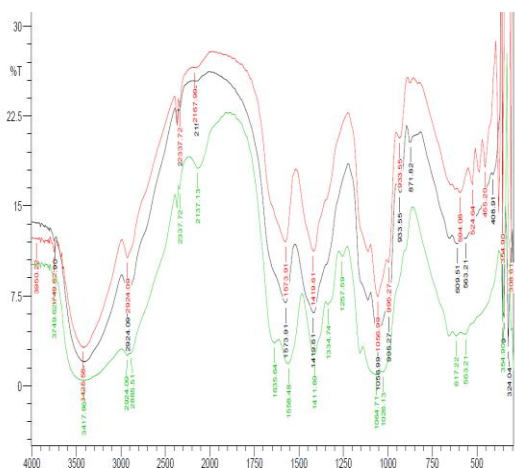
**Figure-1.** FTIR spectra of bacterial cellulose-glycerol/BC-G (upper), BC (medium), and bacterial cellulose-glycerol-chitosan/BC-G-Ch (lower).

Table-2 shows absorbance of $-\text{OH}$ and $-\text{NH}$ 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 $-\text{OH}$ group. The decreasing absorbance of $-\text{OH}$ 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 $-\text{OH}$ group on the spectrum of BC at around the 3400 cm^{-1} .

The broadening of this band indicates an overlapping between $-\text{OH}$ and $-\text{NH}_2$ groups due to the hydrogen bond [7]. Based on Figure-2, the presence of the bands at 635.64 cm^{-1} and 1566.20 cm^{-1} associated with the stretching of $\text{C}=\text{O}$ (Amide I) and bending of $-\text{NH}$ (Amide II), respectively, suggests that the amide group of chitin are 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 | $-\text{OH}$ | 0.360 | 0.320 | 0.329 |
| 1635 | $-\text{NH}$ | - | - | 0.116 |

Morphological images

Figure-2 shows morphological image of BC, BC-G composite, and BC-G-Ch. Bacterial cellulose is composed by fibrillar 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 shows bulk 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.

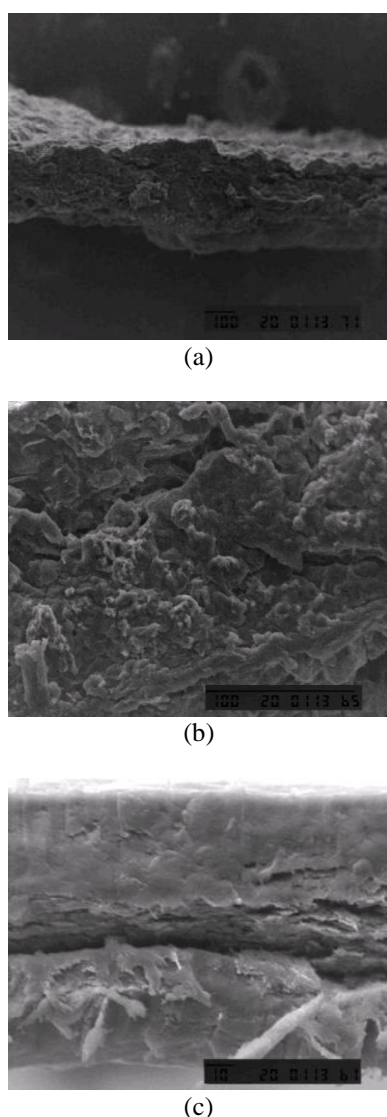


Figure-2.(a) Cross section photo of BC (100x), (b) surface photo of bacterial cellulose-glycerol composite (100x), (c).cross section photo of bacterial cellulose-glycerol-chitosan composite (100x).

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 is evident that the addition of glycerol as a plasticizer reduces the tensile strength but increases elongation of bacterial cellulose. Rechia *et al.* [15] 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 [10] 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 lowest 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 [14]. 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 [15].

The existence of rigid structure in the polymer chain will make it difficult to move when it is pulled so that elongation will decline [10]. 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 to its amorphous nature. The BC has a high crystallinity, which related to high mechanical properties of BC [3, 11]. Zhijiang [11] studied that the addition of chitosan on BC with the concentration range from 12 to 45 wt % decreased its tensile strength from 130 MPa to 54 MPa 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° is associated with the phases of cellulose 1α (1001α , 1101β , and 0101β), nevertheless peak at 22.5° reveals the phases planes of cellulose 1β (2001β) [2]. The BC shows diffraction peaks at above 22.5° . For X-Ray diffractogram of BC-G and BC-G-Ch at above 30° , the broadening peak corresponding to amorphous phase was observed. It indicates that the addition of glycerol and chitosan can change crystalline region in BC.

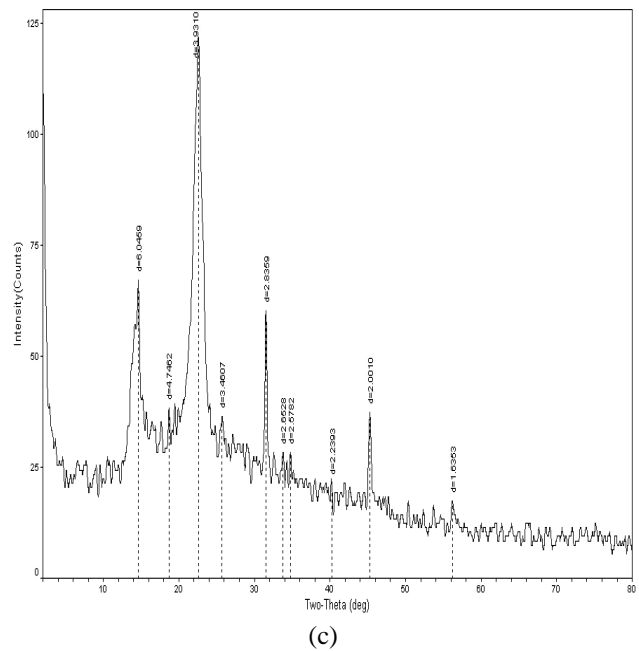
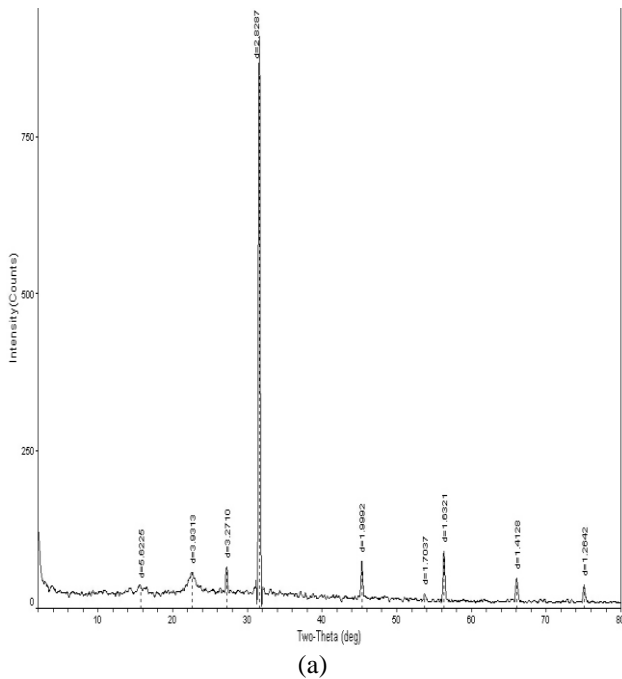
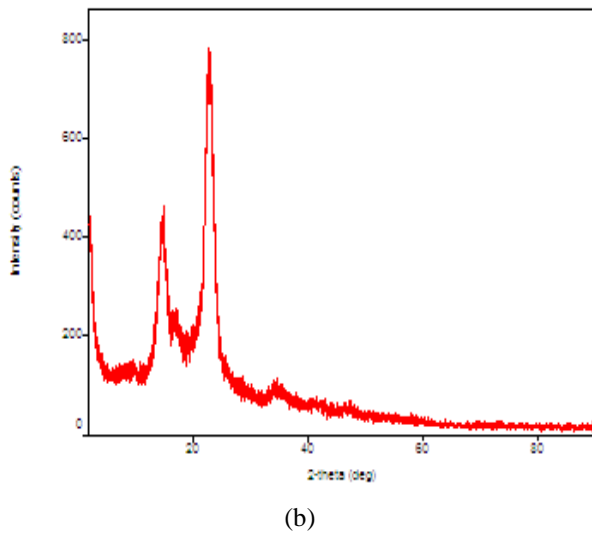


Figure-3. The X-Ray diffractogram of (a) BC; (b) BC-G; and (c) BC-G-Ch.



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 [15]. The decreasing degree of crystallinity can decrease tensile strength. The BC-G-Ch exhibits 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 at elevated 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.

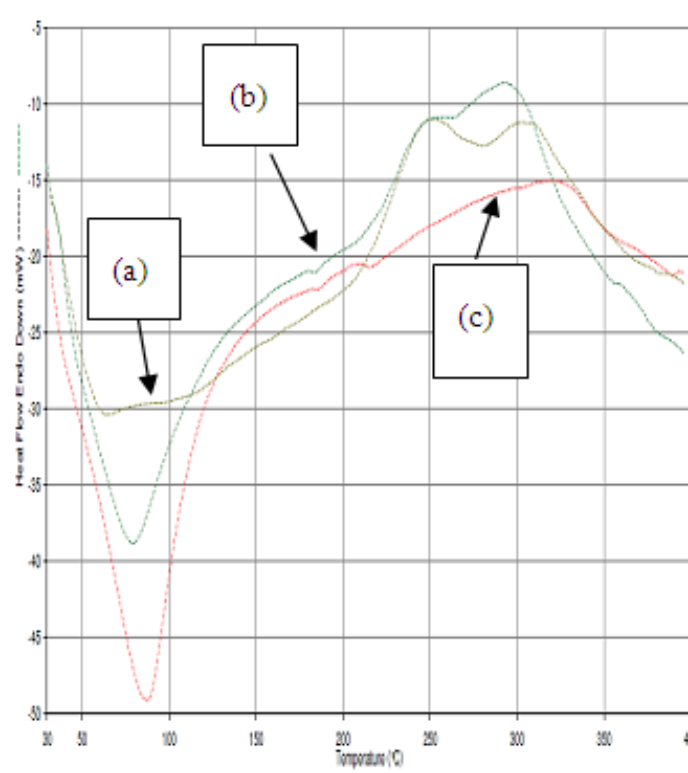


Figure-4. The DTA thermogram of BC (a); BC-G (b); and BC-G-Ch (c).

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 pyrolysis, and it can be observed as a broad exothermic peak [2]. Glass transition of BC was observed at 270 °C and crystallization of its was observed at 330°C [2]. The BC and its composites were degraded at above 400°C.

As shown in Figure-5, two significant losses of weight were observed at the temperature range of 37-100°C and 200-350°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°C for membrane dehydration.

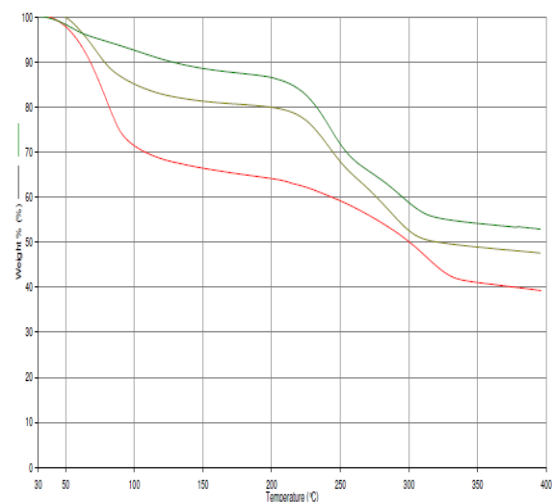


Figure-5.The TGA thermogram of BC-G (upper), BC (medium), and BC-G-Ch (lower).

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 [16]. 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 200°C temperature [12]. 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 cellulose 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 [19]. 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 [20] that glycerol with higher concentration exhibits 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 [20, 21]. 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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