Preliminary study of the degradation of biocellulose based film using soil fungi Aspergillus unguis TP3 and Paecilomyces marquandii TP4 producing cellulose

Y Srikandace*, D G S Andayani and M Karina

Research Unit for Clean Technology, Indonesian Institute of Sciences (LIPI), Komplek LIPI Bandung, Jalan Sangkuriang, Gedung 50, Bandung 40135, Indonesia

*E-mail: yoice_srikandace@yahoo.co.id

Abstract. Bacterial cellulose or biocellulose (BC) is cellulose produced from the activity of bacteria in the suitable growth media containing glucose as mainly carbon source. Due to its unique properties, BC is used for edible packaging. Many studies reported on anti bacterial activity on BC based-edible packaging against Escherichia coli and Staphylococcus aureus as well as Listeria monocytogenes. This study reports the biodegradation of BC (sample A) based-film by using Aspergillus unguis and Paecilomyces marquandii. For comparison, BC film was added with CMC (sample B), with glycerol (sample C), with CMC and glycerol (sample D), respectively. Biodegradation was carried out using broth fermentation and solid substrate fermentation (SSF). BC films (1 cm x 1 cm) and fungi were fermented in 100 mL of sterile aquadest for 60 days with agitation (120 rpm at room temperature). For treatment in SSF, fungi were inoculated into BC films for 60 days at room temperature in petridish. Results showed that all compounds of films were still available based on FTIR results. The physical performance of films BC was in solid chewy (A), like chewy thread (B and C), and like powder (D) form. Results showed that both fungi biodegraded films through broth fermentation whereas no biodegradation activity on SSF. From SEM analysis, it showed that the film surfaces performed fine and smooth morphology.

1. Introduction

Cellulose is the most common organic polymer in nature and has a tremendous economic importance globally. It usually produces from vascular plants such as wood and cotton. However, for its isolation and purification, various chemical treatments are required which is not only time and energy consuming but also create the new environment problems [1]. Other than from plant resources, cellulose can be produced by the activity of bacteria in the suitable growth medium containing mainly of glucose [2]. Various non-toxic and gram negative bacteria such as Gluconacetobacter xylinus, formerly known as Acetobacter xylinum, secretes a large quantities of cellulose as microfibrils from a row of synthetic sites of cells using the enzymes of cellulase system [3]. So far, this bacteria is the most effective and efficient for cellulose production. Cellulose is derived from fermentation process. Microbial polysaccharide composed by cellulose fibers are produced by strains xylinum, Acetobacter aceti subspecies of a non-pathogenic bacteria. Due to cellulose production, the bacteria named as cellulose or bacterial cellulose (BC) [2,4].

Due to its unique and excellent properties, BC can be used in a wide range of applications such as for flexible conductive polypyrrole nanocomposite membranes, electrically conductive nano graphite
composites [5], adsorbent and catalyst [6], wound dressing, drug delivery, tissue engineering [7] and edible packaging [8].

For edible packaging, BC has been evaluated for its anti-bacterial activity against *Escherichia coli* as gram-negative bacteria and *Staphylococcus aureus* as gram-positive bacteria as well as *Listeria monocytogenes* [9,10]. However, study on the degradation of BC with fungi is not discussed in detailed yet. Fungi are the main microorganisms to produce cellulose and to conduct the biodegradation [9]. It has been reported that several fungi such as *Trichoderma* spp, *Aspergillus* spp, *Aspergillus chaetomium*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Curvularia*, *Fusarium*, *Memoniella* sp, *Phomo* sp, *Thielavia* sp and *Penicillium* spp degraded cellulose [9,11,12]. In a previous study, soil fungi *Aspergillus unguis* TP3 and *Paecilomyces marquandii* TP4 were isolated from Tangkuban Perahu Mountain, West Java, Indonesia and investigated for their antibacterial activity for *S. aureus*, *Enterococcus* sp and *E. coli*, and cytotoxic activity for T47D cells [13]. The current study aims to evaluate the BC films degradation using fungi TP3 and TP4.

2. Materials and methods

2.1. Materials and preparation of Bacterial Cellulose (BC) based film

BC in the form of nata-de-coco gel was purchased from the local small scale industry in Cianjur, West Java, Indonesia. The washing, purification, and preparation of BC film slurry were conducted as previously reported [14]. A total of 0.5 kg gel was blended with 200 mL water for 15 min in order to obtain the BC slurry. An amount of 100 mL BC slurry was put in a beaker glass, stirred at 60°C for 10 min, de-gassed using vacuum bell jar to remove bubbles, poured into the Teflon coated-tray, and kept in an oven blower at 45°C for 24 hr until it completely dry in order to produce sample A. By the same treatment, each of BC slurry was added with 0.1 g CMC (sample B), with 200 µL glycerol (sample C), with CMC and glycerol (sample D). The composition of the mixture is shown in Table 1.

| Sample code | Mixture                  |
|-------------|--------------------------|
| A           | BC slurry                |
| B           | BC slurry + CMC          |
| C           | BC slurry + glycerol     |
| D           | BC slurry + CMC + glycerol|

2.2. Biocellulose based films degradation

Fungi *A. unguis* TP3 and *P. marquandii* TP4 were regenerated in Potato Dextrose Agar plates (PDA, Himedia) for 7 days. For pre-treatment, three plugs fungus size 1 x 1 cm from PDA plate were inoculated into 50 ml Potato Dextrose Broth (PDB, Himedia) and fermented for 7 days at room temperature on shaker with agitation 150 rpm. For broth fermentation, three pieces of each film samples (film A, B, C, D) sized 2 x 2 cm and 5 mL culture fungus from PDB media were inoculated into 100 mL sterilized water and fermented for 60 days with same condition with pre-treatment. For solid substrate fermentation (SSF), three plugs fungus sized 1 x 1 cm from PDA plate were put on film sample sized 2 x 2 cm and incubated at 25°C for 60 days in a incubator. For control, each of films (2x2 cm) without fungus was inoculated into 100 ml sterilized water and fermented as well. All fermentations or treatments were performed with three replicates and degraded films were analyzed.
2.3. Cellulase activity of fungi
An amount of 250 mL of Czapek-Dox broth medium containing of 1% CMC was adjusted to pH 5. Three plugs of fungus (1 x 1 cm) were inoculated, fermented and incubated with agitation 120 rpm, 25°C for 7 days. The resulted mycelia were removed whereas the supernatant as crude enzyme was collected for cellulase activity by using Dinitrosalicylic acids (DNS) method. One millilitre of crude enzyme filtrate was added to 0.5 ml of 1% CMC in 0.05 M phosphate buffer pH 6.5 in a test tube and incubated at 50°C for 30 min. After incubation, 1.5 mL of the DNS reagent was added to terminate the reaction. The tube was boiled at 100°C for 10 min and monitored for colour development. Optical density readings were taken at 546 nm and the sugar released extrapolated from the standard glucose curve. The cellulase activity (U/mL) was defined as the quantity of the enzyme that produced 1 µM of glucose per min under the assay conditions [15,16].

2.4. Macroscopic and microscopic morphology of BC films
The macroscopic morphology of all films were photographed with digital camera. For microscopic structure, BC film and biodegraded films were examined for its surface characteristics using SEM JEOL JSM IT-300 JAPAN, operated at 20kV. Film specimens were mounted on aluminum stubs using double-sided tape and then coated with a layer of gold (40–50 nm), allowed to surface visualization.

3. Results and discussion

3.1. Macroscopic and microscopic morphology of biocellulose based films

According to Figure 1(a), BC film showed the rough surfaces associated to parts of cellular fibers. BC has been known to produce highly pure fiber network structure. The pure fibers was due to the Acetobacter xylinum fermentations result using coconut water medium [2,3]. Addition CMC into BC film in Figure 1(b) produced a lot of air bubble, it becaused of the dissolution process between CMC with BC not quite homogenous. CMC also provided the compact structure of the BC film as well. The studies of cassava starch and sorghum starch based films resulted that the addition of CMC increased tensile strength and reduced elongation at break of the blended films [16,17]. Meanwhile, BC film Figure 1 (c) containing glycerol appeared more compact structures but the surface film was not smooth and flat. The mixture BC and glycerol resulted in improvement of films flexibility and decrease tensile strength and increase elongation at break. The study of edible film from plum gums that plasticized with glycerol (5%, 10%, 15%, and 20% w/w) showed the increasing elongation at break in line with increasing the glycerol concentrations [14,18]. In Figure 1(d), the cellulose fiber of BC film consisted of CMC and glycerol described a smooth and rigid structure even the CMC still appeared.
The polysaccharides or cellulose incorporation with CMC and glycerol significantly improved the polysaccharides skeleton and provided an enhancement the physicochemical properties of the film [17,19].

In this study, uniformity and microstructural characterizations of films were assessed using scanning electron microscopy (SEM). The BC films in Figure 2(a) showed the fibrous structures clearly that formed networks structures. Addition of CMC and glycerol solution during the film formation organized themselves and formed homogenous fibrous. The films (Figure 2b and 2c) appeared homogeneous and compact structures with micropores. On the other side, BC film incorporated with CMC-glycerol (Figure 2d) revealed the the smooth and homogenous fibers and also reduced pores.

The interaction among BC, CMC and glycerol improved the physical and mechanical properties of BC-based films and produced the smooth films. A study of cassava starch-CMC films showed SEM micrographs confirmed homogeneity [17]. Those additives influenced the water solubility and density of BC composite films compared to the control (BC film) [14].

3.2. Cellulase activity of fungi and biodegradation films

In nature, BC films were degraded by fungi and bacteria due to the ability of microorganism producing cellulase. Fungi are widely used in biodegradation studies because the fungi are the sources of diverse enzymes. In present study, the cellulase activity of fungi *A. unguis* TP3 and *P. marquandii* TP4 is described in Figure 3.

These BC films have been proposed to replace or complement conventional packaging due to its natural degradation properties. The fungi did not grow neither in BC film nor in film added with CMC and glycerol on solid substrate fermentation (SSF). On the other side, fungi could biodegrade films through broth fermentation. Some previous studies also showed that fungus *P. variotii* NFCCI 3343 as a common wood degrader obtained the cellulolytic enzymatic activity 1.70 U/mL (Czapek medium) and 0.117 U/ml (CMC medium) [20,21]. Fungus *A. unguis* also produced a high activity of cellulase 48.86 U/mL in carbon source (rice bran) on cellulase production [22]. Fungus *A. niger* showed cellulase activity (2.00 U/mL) in Czapek medium and contained total protein 496,60 mg/mL [15]. Fungi *A. niger* was also used for cellulase production in submerged (SmF) and solid state fermentation (SSF) [23]. It indicated that fungi are the main cellulase-producing microorganisms. The various value of enzyme activity of fungi, might be due to composition carbon source, duration of fermentation, temperature and pH condition. In order to increase the cellulase activity, the combined effect of all the growth factors still need to be observed.
Figure 3. Cellulase enzyme production of fungi.

Figure 4 (a). BC film.  Figure 4 (b). BC film added with CMC.  Figure 4 (c). BC film added with glycerol.  Figure 4 (d). BC film added with CMC and glycerol.

Figure 5 (a). BC film.  Figure 5 (b). BC film added with CMC.  Figure 5 (c). BC film added with glycerol.  Figure 5 (d). BC film added with CMC and glycerol.
All degraded films using fungi *A. unguis* (Figure 4) and *P. marquandii* (Figure 5) were shown SEM. The microscopic images of degraded films containing CMC and glycerol (Figure 5d) still showed the smoother threads than the threads of films composed with glycerol (Figure 5c). Films (Figure 5a) and (Figure 5b) still obtained cracks, rough thread and pores. The biocellulose contained CMC had a compact and wavy structure and CMC-glycerol film presented a smooth-homogeneous structure in the film matrix [21,22]. There was no difference in weight between films and degraded films in this study. There are only differences in the surface of a rather stiff film that becomes softer. Under SEM, all films fibers clearly appeared the forming a network that is strongly bound together. It can occur because the functional groups of biocellulose fibers consist of strong C-H and COO-bonds. The fiber length also cannot be determined because the single fibers are interconnected with each other forming a network structure [4]. The film degradation process by fungi for 60 days turned out to be unable to break the C-H and COO-bonds found in cellulose fibers. However, the process was able to change the surface of the films to be smoother and softer so that it is expected to be easily degraded in nature which contains many decomposing microorganisms.

4. Conclusion
Fungi *A. unguis* and *P. marquandii* were capable of producing cellulose which is expected to degrade cellulose. Biodegradation of biocellulose based films in liquid fermentation of soil fungi for 60 days was able to change the rigid surface of BC-film into elastic form.

Acknowledgement
The authors greatly acknowledge the funding of the independently research project of LIPI 2018 and Insinas 2018 of Ristekdikti. The authors also thanked to the collaboration of staffs and supply the material.

References
[1] Kim S S, Lee S Y, Park K J, An H J, Hyun J M and Choi Y H 2017 *Saudí J. Biol. Sci.* 24 314–9
[2] Keshk S M 2014 J Bioprocess. Biotechniq. 4 150
[3] Gupta P, Samant K and Sahu A 2011 *Int. J. Microbiol.* 2012 578925
[4] Mohammad S M, Rahman N A, Khalil M S and Abdullah S R S 2014 *Adv. Biol. Res.* 8 307–13
[5] Chen J, Xu J, Wang K, Qian X and Sun R 2015 *ACS Appl. Mater. Interfaces* 7 15641–8
[6] Wang J, Lu X, Ng P F, Lee K L, Fei B, Xin J H and Wu JY 2015 *J. Colloid Interfere. Sci.* 15 32–48
[7] Sahana T G and Reka P D 2018 *Mol. Biol. Rep.* 45 2857–67
[8] Guo C and Hongwen C 2014 *Food Hydrocoll.* 35 476–83
[9] Petr B and Vendula V 2008 *FEMS Microbiol. Rev.* 32 501–21
[10] Makesh Kumar V and Mahalingam P U 2011 *IJPBA* 2 1695–8
[11] Wilson D B 2011 *Curr. Opin. Microbiol.* 14 259–63
[12] Asma H, Archana S, Sudhir K J, Rakesh K B, Sangeeta R and Mukesh K A 2012 *ABR* 3 10–7
[13] Andayani D G S, Sukandar U, Sukandar E Y and Adnyana I K 2015 *HAYATI J. Biosci.* 22 186–90
[14] Indrartri L, Indriyatni, Syampanywadi A and Pujiasutti S 2016 *AIP Conf. Proc.* 1711 050007
[15] Patil K C, Patil A M, Wagh M C and Chandratre S J 2015 *Res. J. Recent Sci.* 4 124–6
[16] Irfan M, Asma S, Quratulain S and Nadeem M 2012 *TJB* 37 287–93
[17] Priyanka P, Yuvraj C, Farha S and Aranganathan V 2012 *IJLPR* 7 56–61
[18] Nhu B M, Duy Q N and Phu H L 2017 *IJMER* 7 31–6
[19] Hishikawa Y, Togawa E and Kondo T 2017 *ACS Omega* 2 1469–76
[20] Polleto M, Heitor L O and Ademir J Z 2014 *Materials* 7 6105–19
[21] Nemazifard M, Kavoosi G, Marzban Z and Ezedi N 2017 *Int. J. Food Prop.* 20 1501–14
[22] Sanyang M L, Sapuan S M and Sahara J 2016 *J. Food Sci. Technol.* 53 326–36