บทความวิจัย

การเตรียมผงแบคทีเรียเซลลูโลส สำหรับวัสดุทางการแพทย์: ส่วนที่ 1

ณรงค์ ลุมพิกานนท์[1*] พรเพ็ญ ศิริดำรง[2] ธนิสร์ วัยโรจนวงศ์[2]
ฉวีวรรณ พูนธนานิวัตกุล[3] กฤต เรืองโสภาพันธ์[3] ราชพร สีจันทร์[4]
และ อาจินต์ บุญเรือง[5]

บทคัดย่อ

งานวิจัยนี้ศึกษาผลของการเตรียมผงแบคทีเรียเซลลูโลส ต่อสมบัติทางกายภาพ ทางเคมีและทางชีวภาพ โดยหมักด้วยน้ำตาลกลูโคสจากข้าวไทย สารสกัดจากยีสต์ น้ำกลั่นและ Acetobacter xylinum TISTR 975 เป็นเวลา 7 วัน จากนั้นจึงเตรียมเป็นผงแบคทีเรียเซลลูโลส ด้วยวิธีที่แตกต่างกัน ได้แก่ การใช้น้ำ สารละลายโซเดียมไฮดรอกไซด์ และบดเปียก และแช่เย็นที่อุณหภูมิ -20 องศาเซลเซียส แล้วทำให้แห้ง ที่อุณหภูมิ -60 องศาเซลเซียส นาน 3 วัน จากนั้นจึงบดด้วยเครื่องบดความเร็ว 20,000 รอบต่อนาที จนได้ผงละเอียด นำไปวัดลักษณะของผงแบคทีเรียเซลลูโลสด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด วัดการกระจายตัวของอนุภาค และการวิเคราะห์การเลี้ยวเบนรังสีเอ็กซ์ พบว่าผงที่ได้จากทั้งสามวิธีมีโครงสร้างระดับนาโนเมตร/ไมโครเมตร ผลจากการวิเคราะห์การเลี้ยวเบนรังสีเอ็กซ์ แสดงลักษณะช่วงพีคที่ 2θ ที่ 15° และ 22° ซึ่งผงแบคทีเรียเซลลูโลสที่เตรียมทั้ง 3 วิธี มีขนาดอนุภาค 32 และ 1449 ไมโครเมตร ตามลำดับ จากผลการตรวจสอบความเป็นพิษด้วยวิธี MTT ของผงแบคทีเรียเซลลูโลสที่เตรียมได้พบว่าเซลล์มีชีวิตรอด 97% ผลการวิจัยที่ได้แสดงว่าผงแบคทีเรียเซลลูโลสที่เตรียมโดยการใช้น้ำและการบดเปียกสามารถใช้เป็นวัสดุทางการแพทย์สำหรับใช้ในงานวิจัยต่อไป

คำสำคัญ: ผงแบคทีเรีย เซลลูโลส; *Acetobacter xylinum*, แบคทีเรียเซลลูโลสที่ผ่านการปรับสภาพ

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[1]สำนักวิชาทันตแพทยศาสตร์ มหาวิทยาลัยแม่ฟ้าหลวง 333 ต.ท่าสุด อ.เมือง จ.เชียงราย

[2] ศูนย์เชี่ยวชาญนวัตกรรมหุ่นยนต์และเครื่องจักรกลอัตโนมัติ สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย

[3] ศูนย์พัฒนาและวิเคราะห์สมบัติวัสดุ สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย

[4] สำนักงานวิจัย คณะทันตแพทย์ศาสตร์ มหาวิทยาลัยมหิดล

[5] ศูนย์เชี่ยวชาญนวัตกรรมวัสดุ สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย

*ผู้นิพนธ์ประสานงาน, e-mail: narong.lum@mfu.ac.th

Preparation of Bacterial Cellulose Powder for Medical Materials: Part I

Narong Lumbikananda[1*], Pornpen Siridamrong[2], Thanit Vairojanawong[2], Chaweewan Poonthananiwatkul[3], Ghit laungsopapun[3], Ratchaporn Srichan[4] and Arjn Boonruang[5]

ABSTRACT

In this research, effects of preparation techniques of bacterial cellulose (BC) powder on physical, chemical and biological properties were investigated. The BC sheets were prepared by fermentation with glucose of Thai rice, yeast extract, distilled water and *Acetobacter xylinum TISTR 975* for 7 days. The BC sheets were further treated by three different techniques including water, NaOH and wet grinding treatment. The BC was neutralized, squeezed out water and kept in –20°C. The BC frozen-sheets were lyophilized at -60°C about 3 days. The BC dry-sheets were finally pulverized at 20,000 rpm until fine powder was obtained. The characterizations of BC powders were measured by Scanning Electron Microscopy (SEM), particle size distribution, and X-ray Diffraction analysis (XRD). SEM micrographs of BC powder obtained from all three treatment methods revealed nano- /micro- fibrils structures. XRD pattern showed two principle diffraction peaks of 2□ at 15v and 22.5° where particle size of water treated BC and wet grinding treated BC powder were about 190 and 716 μm, respectively. The MTT cytotoxicity of the obtained BC powder was studied using Human Gingival Fibroblast (HGF) at passage 6. The results showed 97% cell viability which is the same to that of commercial product. The results were supported that BC powders prepared from water treated and wet grinding methods can be used as medical materials for the next research.

Keywords: Bacterial Cellulose powder; *Acetobacter xylinum*; Treated bacterial cellulose

[1]School of Dentistry, MEA FHA LUANG University, 333 M.1 Mueang Chiang Rai, Chiang Rai 57100, Thailand

[2]Expert Centre of Innovation Industrial Robotics and Automation , Thailand Institute of Scientific and Technological Research, Khlong Luang, Pathum Thani, 12120, Thailand

[3]Material Properties Analysis and Development Center, Thailand Institute of Scientific and Technological Research, Khlong Luang, Pathum Thani, 12120, Thailand

[4]Research office, Faculty of Dentistry, Mahidol University, Yothi Road, Rajthawee District, Bangkok, 10400, Thailand

[5]Expert Centre of Innovation Material , Thailand Institute of Scientific and Technological Research, Khlong Luang, Pathum Thani, 12120, Thailand

*Corresponding author, e-mail: narong.lum@mfu.ac.th

1. Introduction

One of the most abundant biodegradable and biocompatible natural polymers is cellulose which gains much considerable attention for medical applications. Particularly, bacterial cellulose (BC) is a renewable and biocompatible generally cellulose produced from the fermentation of bacteria. Due to its biocompatibility and biodegradability, BC provides several potential applications including wound dressing [1, 2], artificial blood vessels [3] and scaffold for tissue engineering [4], etc. Among various types of bacteria *Acetobacter xylinum* is the most popular non-photosynthetic organism using glucose, sugar and other substrates to produce cellulose. *Acetobacter xylinum* is a Gram-negative, rod shaped and aerobic bacterium. Properties of BC differs from regular plant cellulose in many aspects including network structure, high water absorption, high mechanical strength and biocompatibility [5]. The appearance of final BC films mainly depends on glucose in which brownish BC powder is normally obtained. To remove impurities and whiten BC, treatment method is required using chemical agents. Sodium hydroxide treatment method having a concentration ranging from 0.1-1 molar, a temperature of 60-100 ^{o}C for 1-3 hours is conventionally applied and BC is then neutralized by acid or excess water [6-8]. Such methods are more complex, costly and the residue sometime causes cell toxicity.

This research aims to produce BC powder with different treatment techniques including water treated and wet grinding BC powder and compared to the conventional chemical treatment. The glucose for fermentation was from Thai rice as source. Physical and biological properties of those prepared BC powder are characterized for further use in medical application. The properties are also compared to those of commercial BC powder form Avicel PH-101.

2. Materials and Methods

2.1 Materials

Materials used in this research were glucose which was fermented from Thai rice, yeast extract, alcohol and sodium hydroxide (NaOH, laboratory grade, pellets) which was supplied by Sigma-Aldrich, USA. *Acetobacter xylinum TISTR 975* was obtained from Thailand Institute of Scientific and Technological Research.

2.2 Preparation of BC sheets

BC sheets were obtained from fermentation of *Acetobacter xylinum TISTR 975*. The cultures were incubated for 7 days at a room temperature in a culture dish. The culture mediums were composed of glucose, yeast extracts and alcohol. After 7 days of incubation, hydrated BC pellicles having 3 mm of thickness containing up to 99% of water and 1% of cellulose were obtained.

2.3 Preparation of BC powder

2.3.1 Water treated BC powder

BC sheets were rinsed with water for 6 hours then neutralized by DI water. After that, the sheets were squeezed to get rid of water and kept frozen at a temperature of −20°C and further dried for 4 days. The BC powder was obtained using high speed grinder at a speed of 20,000 rpm.

2.3.2 NaOH treated BC powder

BC sheets were boiled in 0.1 N NaOH at a temperature of 100°C to remove bacteria for 2 hours. After that, the BC sheets were washed with DI water until neutral pH was achieved. The samples were squeezed to get rid of water and kept frozen at a temperature of −20°C and further dried for 4 days. The BC powder was obtained using high speed grinder at a speed of 20,000 rpm.

2.3.3 Wet grinding BC powder

For wet grinding BC powder preparation, BC sheets were rinsed with water for 6 hours and ground into powder using high speed grinder at a speed of 35,000 rpm. The ground samples were freeze at a temperature of −20°C and further dried for 4 days. The BC powder was obtained using high speed grinder at a speed of 20,000 rpm.

3. Characterization of BC

3.1 Physical properties

The BC sheet and BC powder were examined by Scanning Electron Microscope, SEM (JEOL, JSM-6400, Japan). X-ray diffraction (XRD) patterns were obtained using nickel filtered Cu K-α-radiation from 20 to 80 (Shimadzu, XRD -6000/7000). The particle size of the obtained BC powder and their distribution were analyzed by Mastersizer 2000.

3.2 Biological properties

In vivo testing of BC powder by MTT cytotoxicity test was performed according to ISO 10993-5:2009. The cell Human Gingival Fibroblast (HGF) passage 6 at a concentration of $1x10^5$ cell/cm^3 were seeded into 96-well plates and maintained in culture for 24 h in Dulbecco's Modified Eagle Medium completed medium (DMEM) under 5% CO_2 at a temperature of 37°C until monolayer of cell was obtained. The 0.2 g BC powder was seeded in 0.1 ml of DMEM completed medium and incubated in 5.0% CO_2 at a temperature of 37°C for 24 hours. The extracted samples were separated into two groups i.e. negative control (Thermanox® Plastic coverslips Lot.No. 600562) and positive control (Polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC): RM-A Lot. No. A-152K). 100 µl of extracted sample were seeded into 96-well plates and incubated under 5.0% CO_2 at a temperature of −20°C for 24 hours, >90% humidity. After that the extracted samples were removed from medium, rinsed once with PBS(1x) then added with 50 µl of 0.1% MTT and incubated under 5.0% CO_2 at a temperature of 37°C for 2 hours, > 95% humidity. MTT were washed out once by PBS (1x), added 100 µl of isopropanol and shook for 30 minutes. The cultured HGF were investigated with microplate reader at absorption wavelength of 570 nm.

4. Results and discussion

4.1 Morphology of BC sheets

The morphology of the BC sheet was observed using SEM as shown in Figure 1.

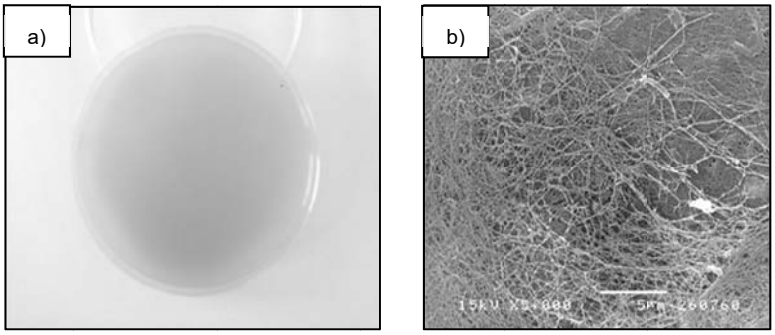

Figure 1 The obtained BC sheet a) physical appearance b) SEM micrograph (magnification of 5,000 X)

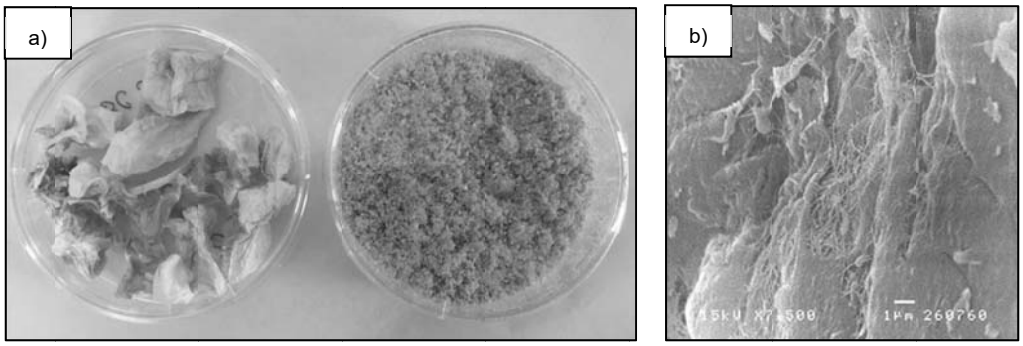

Figure 2 Water treated BC a) dried BC sheet and BC powder b) SEM micrograph (magnification of 7,500 X)

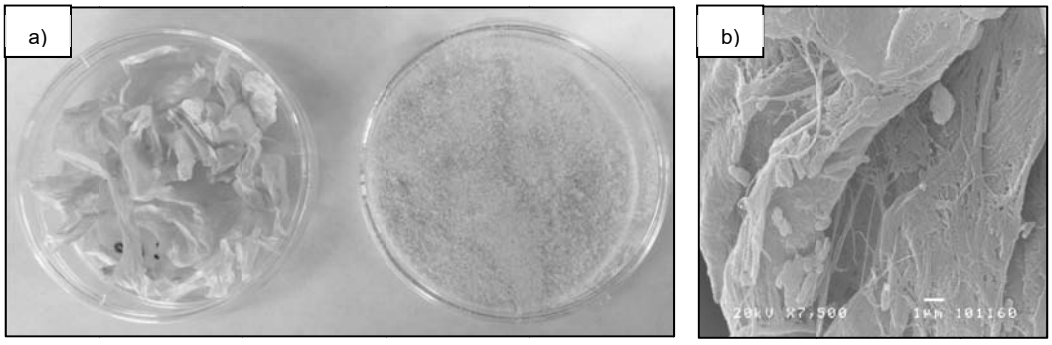

Figure 3 NaOH treated BC a) dried BC sheet and BC powder b) SEM micrograph (magnification of 7,500 X)

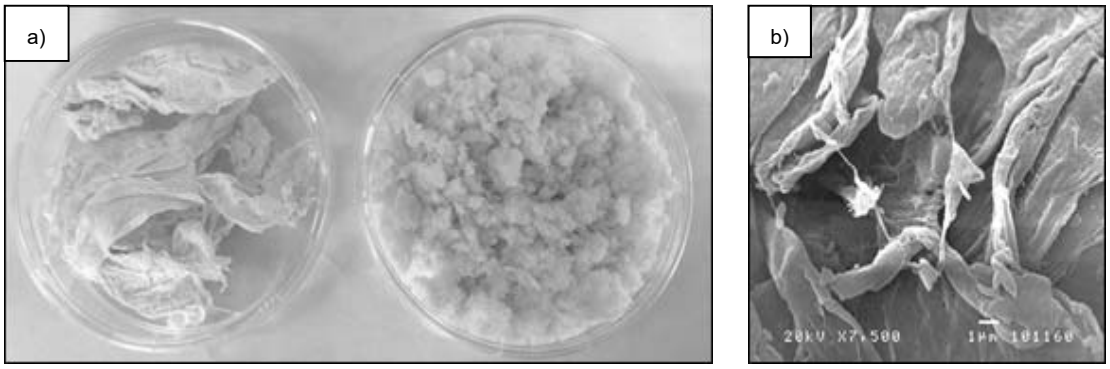

Figure 4 Wet grinding BC a) dried BC sheet and BC powder b) SEM micrograph (magnification of 7,500 X)

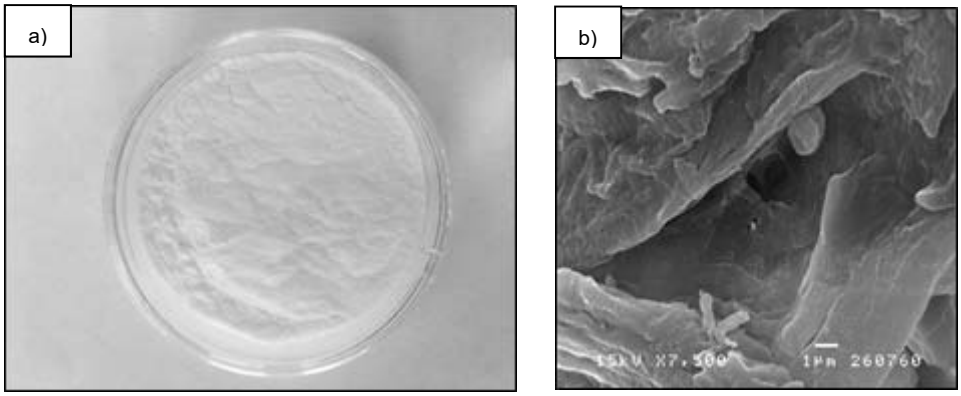

Figure 5 Commercial BC powder from Avicel PH-101 a) dried BC powder b) SEM micrograph (magnification of 7,500 X)

The obtained BC sheet was yellowish translucent and soft as can be observed in Figure 1a). Figure 1b) shows SEM micrograph of BC sheet revealing interwoven mesh of BC fibril network with high porosity. After treated, BC powder exhibited a change in appearance i.e. wet grinding BC powder was white whereas NaOH and water treated BC powder were light-yellow and light-brown, respectively as observed in Figures 2 to 4. Moreover, the difference in morphology of the resulting BC powder was also observed i.e. the fibril network of BC sheet transformed to a dense structure with lower porosity. Figure 4 exhibited remarkable difference in the morphology of BC powder prepared from wet grinding compared to that of BC powder after treated with water and NaOH. The wet grinding BC powder showed tough and soft BC powder whereas those BC powder prepared using water and NaOH was more rigid and flake-like structure. Moreover, the aggregate of BC powder and enlarge particle size from wet grinding was also noticed. In comparison, such micrographs of the prepared BC powder were similar to that of commercial product from Avicel PH-101 as noticed in Figure 5.

4.2 XRD analysis

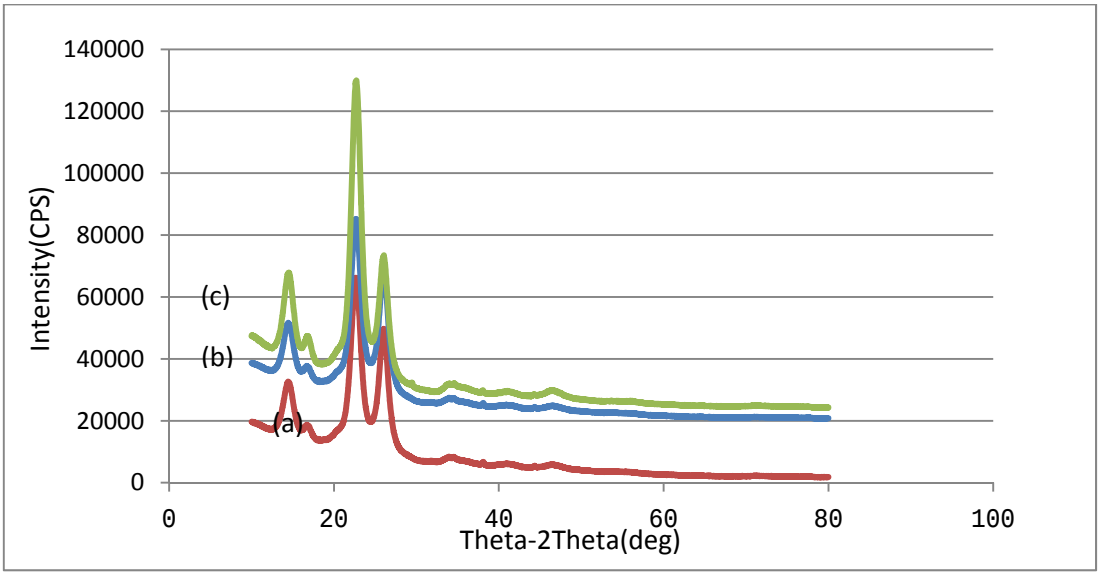

Figure 6 X-ray pattern a) water treated BC powder b) wet grinding treated BC powder c) NaOH treated BC powder produced from *Acetobacter xylinum* TISTR 975.

The XRD patterns of water treated, wet grinding and NaOH treated BC powder are shown in Figure 6. Typical BC crystalline phases were observed in all XRD patterns of water treated, wet grinding and NaOH treated BC powder. Characteristic peaks of BC were identified by diffraction peaks at 15° and 22.5° which assigned to the cellulose Iα and Iβ phases (1001α, 1101β and 0101β planes at 15 ° and 1101α and 2001β at 22.5° [9, 10]. The obtained XRD patterns were similar to that reported for Avicel PH-101 commercial BC powder [11].

4.3 Particle size distribution analysis

Table 1 Particle size distribution of water treated, wet grinding, NaOH treated BC powders and Avicel PH-101 powder.

Samples	Particle size distribution (µm)		
	d (0.1)	d (0.5)	d (0.9)
Water treated BC powder	238.565	779.740	1437.588
Wet grinding BC powder	32.069	190.254	901.995
NaOH treated BC powder	168.947	716.919	1449.370
Avicel PH-101	6.004	27.955	69.768

The particle size distributions of all BC powders are summarized as shown in Table 1. The particle size of water treated and NaOH treated BC powders were similar and bigger than that of wet grinding BC powder. The result was in consistence with the SEM micrograph. Those values of water treated, NaOH treated and wet grinding BC powder were in the range of 238 to 1437, 168 to 1149 and 32 to 901 μm, respectively. Such particle sizes of all BC powders were greater than that of Avicel PH-101 commercial BC powder having the average particle size of 25 μm.

4.4 *In vivo* testing by MTT cytotoxicity test

Table 2. MTT cytotoxicity test of water treated, wet grinding and NaOH treated BC powders.

Samples	% Cell viability	Negative control	Positive control	Control
Water treated BC powder	97.39	97.82	9.78	100
Wet grinding BC powder	98.07	97.95	9.73	100
NaOH treated BC powder	96.02	96.54	9.67	100

MTT results were directly proportional to the number of living cells. As can be seen in Table 2, the cell viability of water treated, wet grinding and NaOH treated BC powder were 97.39, 98.07 and 96.02%, respectively which were greater than 70% for 24 hours and no significant difference was determined. Such results indicated that water treated and wet grinding BC powders showed no cytotoxicity thus those treatment techniques are potential methods to prepare the BC powder for further used in medical application.

5. Conclusion

The effects of treatment techniques for preparation of BC powder on physical chemical and biological properties were observed. The BC sheets were fabricated from glucose of Thai rice and fermented using *Acetobacter xylinum TISTR 975*. The BC sheets were then treated by several techniques including water, NaOH and wet grinding methods. It was found that water treated BC powder and wet grinding BC powder were suitable techniques to prepare BC powder compared to the conventional chemical treatment as results of their appearance and cytotoxicity having 97% cell viability. Such results indicated that water treatment and wet grinding are potential techniques to prepared BC powder for further used as medical material for further research.

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References

1. UI-Islam, M., Shah, N., Ha, J. H., and Park, J. K. 2011. Effect of Chitosan Penetration on Physico-Chemical and Mechanical Properties of Bacterial Cellulose. *Korean Journal of Chemical Engineering.* 28: 1736-1743.
2. Kim, S. Y., Kim, J. N., Wee, Y., Pard, D. H., and Jo, C. 2011 Preparation and Characterization of a Bacterial Cellulose/Chitosan Composite for Potential Biomedical Application. *Journal of polymer research.* 18: 739-744.
3. Putra, A., Kakugo, A., Furukawa H., Gong J. P., and Osada, Y. 2008 Tubular Bacterial Cellulose Gel with Oriented Fibrils on the Curved Surface. *Polymer.* 49: 1885-1891.
4. Kim, J., Cai, Z., and Chen, Y. 2010 Biocompatible Bacterial Cellulose Composites for Biomedical Application. *Journal of nanotechnology in Engineering and Medicine* 1: 1-7.
5. Cai, Z., and Kim, J. 2010. Bacterial Cellulose/Poly(Ethylene Glycol) Composite: Characterization and First Evaluation of Biocompatibility. *Cellulose.* 17: 83-91.
6. Mitrofanov, R., Budaeva, V., and Sakovich, G. 2010. Preparation and Properties of Bacterial Cellulose Gel Films. *Chemistry for sustainable development.* 18: 503-508.
7. Shao, W., Wang, S., Liu, H., Wu, J., Zhang, R., and Min, H. 2016. Preparation of Bacterial Cellulose/Graphene Nanosheets Composite Films with Enhanced Mechanical Performance. *Carbohydrate* Polymers. 138: 166-171.
8. Barud. H. G. O., Barud, H., Cavicchioli, M., Amaral, T. S., Oliveira Junior, O. B, Santos, D. M., Peterson, A .L., Celes, F., Borges, V. M., Oliverira, C. I., Oliveira, P. F., Furtado, R. A., Tavares, D. S., and Ribeiro, S. J. L. 2015. Preparation and Characterization of a Bacterial Cellulose/Silk Fibroin Sponge Scaffold for Tissue Regeneration. *Carbohydrate Polymers.* 128: 41-51.
9. Jai, Y., Huo, M., Zhai, X., Li, F., and Zhong, C. 2017. Preparation and Characterization of a Novel Bacterial Cellulose/Chitosan Bio-Hydrogel. *Nanomaterials and Nanotechnology.* 7: 1-8.
10. Phisalaphong, M., and Sangtherapitiku., P. 2008. Novel Nanoporous Membranes from Regenerated Bacterial Cellulose. *Journal of Applied Polymer Science.* 107: 292-299.

11. Xia, S., Li, H., Ravula, S., and Zhao, H. 2014. Aqueous Ionic and Deep Eutectic Solvents for Cellulosic Biomass Pretreatment and Saccharification. *Royal Society of Chemistry.* 4: 10586-10596.

ได้รับบทความวันที่ 30 พฤศจิกายน 2560
ยอมรับตีพิมพ์วันที่ 17 พฤษภาคม 2561