Pak. J. Biotechnol. Vol. 14 (3) 323-327 (2017)
www.pjbt.org

ISSN Print: 1812-1837
ISSN Online: 2312-7791

THE EFFECT OF POLYSACCHARIDE IN THE FERMENTATION MEDIUM ON PHYSICAL PROPERTIES OF BACTERILA CELLULOSE FROM *Gluconacetobacter xylinus* BTCC B796

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Article received 5.7.207, Revised 21.8.2017, Accepted 29.8.2017

ABSTRACT

Effect of addition of starch, agar, and alginate on the physical properties of Bacterial Cellulose produced by *Gluconacetobacter xylinus* BTCC B796 has been studied. The strain was grown in *Hestrin Schramm* medium supplemented by either starch or agar or alginate with various concentrations at room temperature for 7 days without shaking. The cellulose produced was analyzed for crystallinity index, functional groups, cellulose morphology, tensile strength, water holding capacity, and rehydration ratio. The addition of starch, agar and alginate decreased the crystallinity of Bacterial cellulose, in which the strongest effect was shown by starch. Furthermore, it was observed that the addition of polysaccharide led to decrease in tensile strength, increase in the rehydration ratio and water holding capacity, except agar. Addition of starch gave more pronounced effects than that of agar or alginate.The addition of starch in the medium resulted in decreasing the crystallinity and changes the physical properties greater than agar and alginate

Keywords: Bacterial cellulose, starch, agar, alginate, physical properties

INTRODUCTION

Bacterial cellulose (BC) is composed of D-glucopyranose units connected by 1.4 β-glycosidic bound. The BC is intensively used as a raw material for food (such as dessert, artificial meat), food ingredient (such as thickener, gelling agent, stabilizer, emulsifier, water binding agent), and food packaging (such as in sausage and meat casings) (Shi *et al*., 2014). Cellulose is also a dietary fiber that has many health benefits. Diet sufficient of cellulose can prevent colon cancer, heart attack, and hypertension (Jagannath *et al*., 2008).

Many researchers have paid attention to the BC because of its fascinating and desirable properties. BC has very small fibrils diameter (1.5 nm) cellulose from plant (100 nm) (Khan *et al*., 2007). BC also has high purity, high crystallinity, high mechanical strength, high WHC, and is also able to make 3 dimention network (Chawla *et al*., 2007; Chen *et al*., 2010). These properties make the BC is widely used in industries. However, the high crystalinity of the BC sometimes not desirable such as in nata de coco due to its affects on texture and sensory properties. In addition, the high crystallinity of BC tends to reduce WHC and exhibits poor rehydration after drying (Huang *et al*., 2010; Lin *et al*., 2009). This properties limit the application of the dried BC for stabilizer or thickener.

The crystalinity index of BC may be controlled during fermentation by manipulating the fermentation medium and culture conditions (Ruka *et al*., 2012; Zhou *et al*., 2007). The presence of xylan in the cultural medium was reported to be able to stick at the surface of individual microfibrils, inhibit ribbon formation, and decrease crystal size and ratio of cellulose Iα (Iijima,1991). Furthermore, the addition of polysaccarides or water-soluble agents into the culture medium can also control the assembly and crystallization of glucan chains in the BC (Tokoh *et al*., 2002; Yan *et al*., 2008). Some polysacharides including sodium alginat (Zhou *et al*., 2007; Cheng *et al*., 2009), agar (Bae *et al*., 2004; Cheng *et al*., 2009) and starch (Grande *et al*., 2008) added in the medium, can improve the yield or properties of cellulosa bacterial due to the decreasing crystallinity. The possible mechanisms in decreasing crystallinity of BC were proposed as inhibition of cell to move and or inhibition in the crystallization process (shibazaki *et al*., 1998; Zhou *et al*., 2007; Huang *et al*., 2010; Lin *et al*., 2009). The presence of other water soluble polymers such as carboxy methyl cellulose (Huang *et al*., 2010; Chen *et al*., 2011), hydroxy propyl methyl cellulose (Huang *et al*., 2010), and gelatin (Lin *et al*., 2009) were also reported to give positive effect to WHC and rehydration ratio. However, the effects of starch, agar (from *Gracilaria verrucosa*) (Rosyida *et al*., 2015), and alginate introduced in the medium on the physical properties of the BC including tensile strength, WHC, rehydration ratio, and springiness have not been explored yet.

This works explored the effect of starch, agar, and alginate in the fermentation medium on the crystalinity index and other physical properties of BC production.

2. Materials and Methods

Microorganism and medium: *Gluconacetobacter xylinus* BTCC B796 obtained from biotechnology centre of LIPI culture collection. Medium used was Hestrin Schramm (HS) medium with containing 2.0% (w/v) D-glukosa, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract,0.27% (w/v) Na_2HPO_4 and 0.115% (w/v) citric acid (Hestrin and Schramm, 1954). Glacial acetic acid was used to lower pH medium around pH 5.0. Polysaccharides were added to the medium are sodium alginate with concentration 0%, 0.04%, 0.07%, 0.1%, agar with 0%, 0.05%, 0.01%, 0.15% and starch with concentration of 0%, 0.5%, 1.5%, 2%.

Growth conditions: Seed culture from HS agar was inoculated to 10 ml HS broth for three days at 30 °C under static conditions. The resulting culture was shaken vigorously to release cells from the cellulose pellicle and cell suspension was inoculated into 100 ml HS medium in 500 ml conical flask at a concentrations of 5% (v/v). Cultures was incubated for 3 days at 30 °C under static conditions. The resulting culture was shaken vigorously to release cells from the cellulose pellicle. 5% (v/v) cell suspension was inoculated into 2 L HS medium containing polysaccharides at different concentration. Cultures was incubated for seven days at room temperature under static conditions.

The resulting pellicle was removed from cultures. Pellicles was rinsed with water and boiled in 0.1 N NaOH for 20 min to remove any residual medium. The pellicle produced were washed repeadly/thoroughly using water until the pH of water became neutral.

Crystallinity Index: X-ray pattern measurement was carried out to analyze the change in crystallinity of the BC by shimadzu-600 diffractometer with using Ni-filtered CuK radiation (k = 1.54 Å). The XRD operating voltage and current were 40 kV and 30 mA, respectively. The crystallinity index (CrI) was calculated from diffracted intensity data using Segal *et al*., (1959) method.

Functional Group of BC: FTIR spectra of the BC was measured at wave numbers ranging from 4000 to 400 cm^{-1} using spectrometer Shimadzu-8201 PC.Corp., Japan) and utilizing the KBr (Potassium Bromide) technique for BC preparation.

Morphology of BC: SEM (JEOL JSM6510 LA) was used to observe the topography of the surface and matrix of pellicle. BC was dried and coated with palladium and then observed with a scanning electron microscope.

Tensile strength: Tensile strength of dried cellulose have been measured using Universal Testing Machine ZWICK Z.05-type mechanical tester.

Water holding capacity (WHC): WHC was determined using Jiang *et al*.,(1985). Nata was cut into cubes of equal dimensions, wrapped in filter paper and centrifuged at 5,000 g for 10 min. During centrifugation the water released is absorbed by the filter paper. The percentage ratio of the moisture in the centrifuged nata to the original moisture content provided the WHC.

Rehidration Ratio: This parameter was measured by following the method, proposed by Bodhibukkana *et al*., (2006). BC were weighted (Wwet) then drying. Dried BC was weighted (W-dry) and immersed in deionized water (w/v = 1:2) until the weight of the rehydrated sample (Wrwet) constant (12 hours) . The rehydration ratio, represented the degree of the removal water and was replaced by deionized water, which was calculated as:

Rehydration ratio(%)=(Wrwet-Wdry)/(Wwet-Wdry) x 100%

Statistical Analysis: The data obtained were analyze using spss system, When analysis of variance (ANOVA) revealed a significant effect ($p < 0.05$), data means were compared using a Duncan multiple range test..

Results and Discussion

Crystalinity Index: The adding of starch, agar, or alginate to the fermentation medium decreased crystallinity index of the cellulose produced. The more concentration of the polysaccharide, the more the crystallinity index of the cellulose produced was decreased, although the decrease of the crystallinity index might not related directly to the viscosity of the fermentation medium (Table 1). The crystallinity index of the cellulose produced in HS medium was 71.7% and it decreased to 25.1, 64.2, and 55.3 when the fermentation medium was supplemented with 0.05% starch, 0.05% agar, and 0.10% alginate, respectively. The last three medium had viscosity of 3.0 cP. The addition of starch to the fermentation medium gave more profound effect to the crystallinity index of the cellulose produced that of agar or alginate. The supplementation of alginate gave the least effect to the crystallinity index of the cellulose produced.

Table 1: Crystallininty Index of BC produced by *G. xylinus* BTCC B796 in HS medium containing polysaccharide at different concentration

Polysaccharide (%,w/v)	Viscosity (cP)	Crystallinity Index (%)
Control	2.0	71.7 ± 1.6[a]
Starch 0.5 %	2.1	45.5 ± 7.7[cd]
Starch 1%	2.2	30.3 ± 1.0[de]
Starch 2%	3.0	25.1 ± 9.2[e]
Agar 0.05%	3.0	64.2 ± 0.1[ab]
Agar 0.1%	5.5	59.6 ± 4.6[abc]
Agar 0.15%	9.6	51.2 ± 6.1[bc]
Alginate 0.04%	2.5	68.04 ± 1.9[ab]
Alginate 0.07%	2.8	63.90 ± 1.5[ab]
Alginate 0.1%	3.0	55.34 ± 4.9[abc]

Value with different letters and the same column are significantly different at a = 0:05

According to Shibazaki *et al*., (1998), The decrease of crystallinity of cellulose when it produced in medium containing polysaccharide can inhibition of the cell movement by the polisaccharide. In order to forming the crystalline structure (ordered), requires cell-free motion. The addition of polysaccharide can change medium viscosity and lead to the inhibition of cell-free movement. Inhibition of cell movement subsequently lead to reduction of crystallinity. Based on the movement inhibition of the cell, starch should give smallest inhibition, but the opposite data was observed. It seems that the viscosity is not a dominant factor affecting the decline of crystallinity of cellulose. According to data, at the same viscosity (3.0 cP) for the three polysaccharide, crystallinity index shows the different. The possible reduction in crystallinity was caused inhibition of the aggregation process by the presence of the polysaccharide into medium (Huang *et al*., 2010). BC crystallinity related to intermolecular and intramolecular hydrogen bonds on BC aggregation steps from subfibril, microfibril until forming the pellicle. Polysaccharide was added to the medium physically block or inhibit the aggregation processes that alter change the pattern of intramolecular bonds of cellulose. The Inhibition of polysaccharide dependent or related to differences in the type, size of the molecular weight and the amount of polysaccharide added (Hirai et al., 1998). The smaller the BM molecules able to inhibit the polysaccharide is added at the beginning crystallization (subfibril level) because of its small size (Huang *et al*., 2010). Furthermore the increasing concentration of polysaccharide which are added the greater effect of the physical inhibition of the aggregation process of BC so that further crystallinity process of the microfibrils inhibited. The other reason that the addition of starch resulted in a decreasing in the crystallinity index most likely due to the similarity with the cellulose monomer backbone. Cellulose and starch are composed by glucoses promoting large affinity to bind between starch and cellulose that can change the hydrogen bonding typical of intra and inter glucoses (Gu and Catchmark, 2012).

Decreasing in crystallinity due to the addition of these polysaccharide are also supported by the observation using FTIR and SEM. Furthermore, it is also observed in table 1 that increasing polisaccharides concentrations caused an increase in the viscosity, so that stronger inhibition in bacteria movement was occurred. As a result, less crystalline of the cellulose production were formed (Hirai *et al.,* 1997).

Functional group of BC: The FTIR spectra of the BCs production in various medium are looked similar, where in peaks at 3600-3000 cm^{-1}, 1765-1715 cm^{-1}., around 1400 cm^{-1} and around 900 cm^{-1} and at around 895.86 cm^{-1} are appeared as shown in Figure 1. The absorption at 3600-3000 cm^{-1} attribute of OH group of B.C, representing intramolecular bond of 2OH ---O6, 3O---HO5 and 6O ---HO3 intermolecular bonds (Oh *et al*., 2005). The peak at 1765-1715 cm^{-1} indicated the presence of C=O stretching (Abraham *et al*., 2011; Mandal and Chakrabarty, 2011; Sundari and Ramesh, 2012). The absorption at around 900 cm^{-1} may be from β glycoside bonds in cellulose, and appearance of peak at 895.86 cm^{-1} strongly supports the presence of BC. (Mandal and Chakrabarty, 2011)

The addition of polysaccharide resulted in peak that appears at 3600-3000 cm^{-1} more wide (broader peak), especially for the starch. This peak declining caused by changing into hydrogen bonds then changing in intra-molecular and intermolecular bonds associated with the bacterial cellose hydrogen bonds (Huang *et al*., 2010). The addition of starch resulted in the crystalline area decline most big decline and possible damage caused by hydrogen bonding and the graph shown as broader peak. The Decreasing OH group on observations using FTIR supporting evidence crystallinity decrease of BC (Huang *et al.,* 2010; Chang *et al*., 2012).

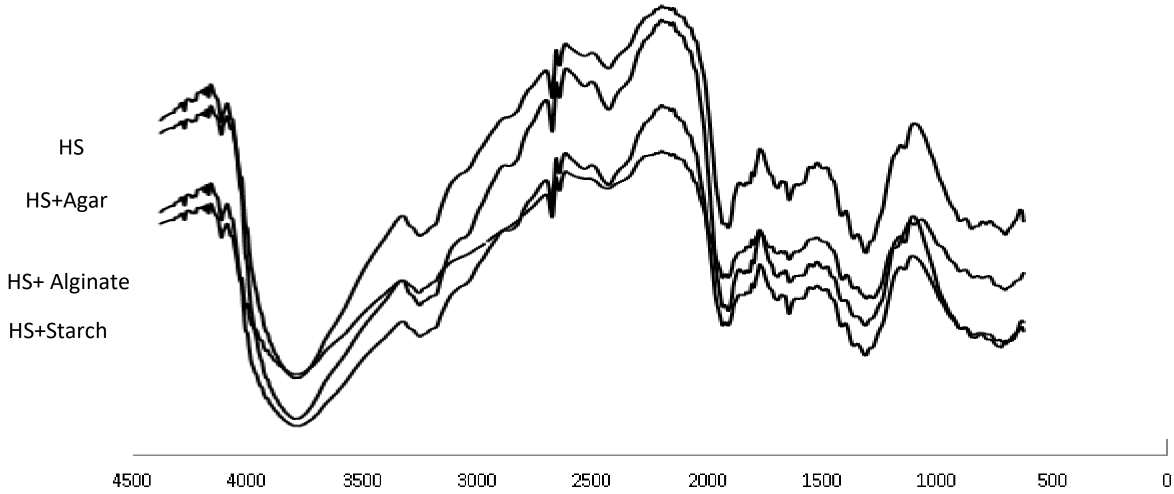

Figure 1. FT-IR spectra of BC produced by *G.xylinus* BTCC B796 in HS medium and HS medium containing starch, agar and alginate

The changing BC structure lead to the changing crystallinity and furthermore affect the tensile strength, WHC and rehydration ratio.

Bakterial cellulose morphology: The SEM images of the BC produced are presented as Fig. 2. The addition of the polysaccharide in the fermentation medium have changed the structure of BC production. The addition of the polysaccharide resulted in microfibril cellulose is covered by their polysaccharide. The addition of starch produce net shaped arrangement of BC and give more void, while adding agar and alginate into medium resulted cellulose bacterial with ribbon shape.

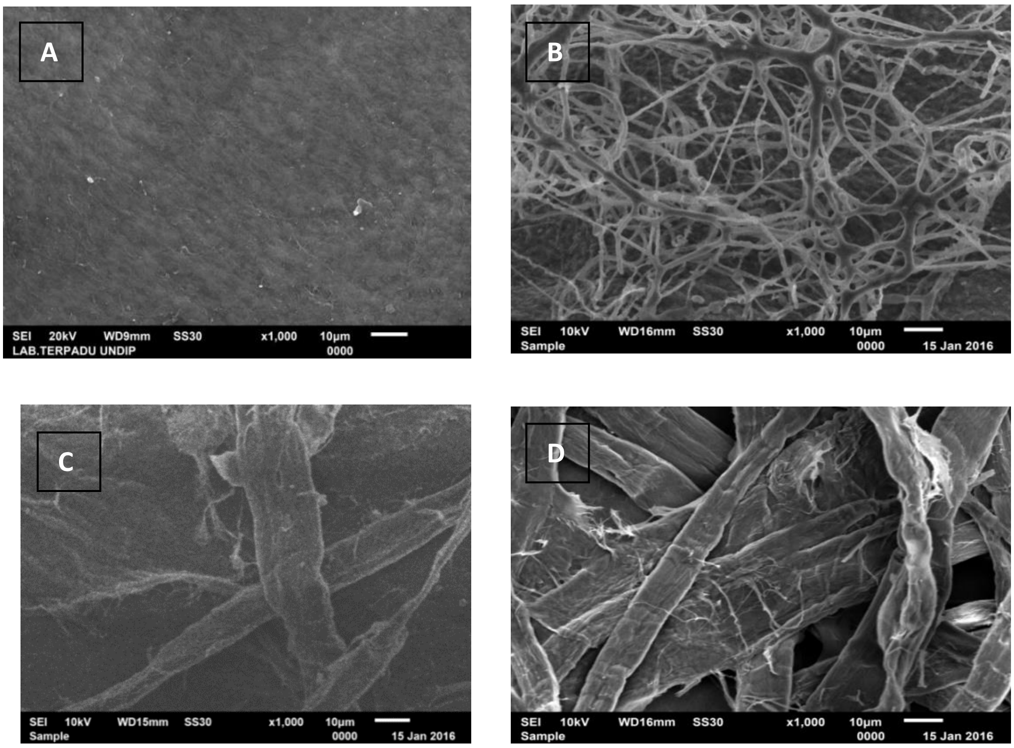

Figure 2: SEM images showing of surface morphologies: of BC in the medium (a) HS (b) HS + 2% Starch, (c),HS+ 0.15% Agar, (d) with 0.10% Alginate

The Changing in relatively dense structure is also evidence for decrease crystallinity cellulose structure which characterized by a dense and regular arrangement (ordered). The addition of additives to the medium can result in a decreasing in the cellulose crystallinity (Lin *et al*., 2009; Cheng *et al*., 2009). The observations uses SEM also shows the addition of agar and alginate in the medium produces cellulose ribbon-shaped with a certain width. The Changing of cellulose structure further more effect on the physical properties of cellulose such as tensile strength and WHC.

Tensile strength: The influence of polysaccharide in the medium on the tensile strength of BC is shown in Table 2. The addition of starch to the medium resulted in decreasing tensile strength compared to medium without addition of polysaccharide, and the tensile strength decrease with increasing the concentration of starch into medium. The addition of agar and alginate in the medium on the initial concentration give tensile strength that greater than the medium without the addition of polysaccharides, but by increasing concentration agar and alginate was added into medium the tensile strength become lower than the medium without the addition of polysaccharides. The addition of starch at a concentration of 2% resulted in decreasing tensile strength greater than all medium. The addition of starch showed a decrease in tensile strength greater than agar and alginate. This is probably related to decreasing the crystallinity index sizeable medium added starch. BC with high crystallinity has a regular arrangement of the cellulose structure and solid resulting from the aggregation of cellulose through hydrogen bonding inter and intra cellulose (Gu and Catchmark, 2008). The observation used FTIR also showed a decreasing in the OH bond of cellulose production primarily by the addition of starch and agar.

Table 2: Tensile strength, WHC and rehydration ratio BC produced by *Gluconacetobacter xylinus* BTCC B796 by adding starch, agar and alginate in the fermentation medium.

Polysaccharide (%, w/v)	Tensile strength (Mpa)	WHC (%)	Rehidration ratio (%)
Control	9.25 ± 1.14^{ab}	81.87 ± 4.89^{abcd}	4.58 ± 1.3^{c}
Starch 0.5%	8.43 ± 5.40^{ab}	87.88 ± 2.13^{a}	5.00 ± 0.56^{c}
Starch 1,0%	6.45 ± 2.92^{ab}	83.52 ± 4.34^{abc}	11.23 ± 1.64^{b}
Starch 2%	4.82 ± 0.74^{b}	82.56 ± 5.90^{abcd}	13.30 ± 1.28^{a}
Agar 0.05%	$9.58\pm1{,}55^{ab}$	77.71 ± 1.25^{cd}	4.34 ± 0.50^{c}
Agar 0.10%	8.44 ± 1.10^{ab}	76.30 ± 2.54^{d}	5.66 ± 1.51^{c}
Agar 0.15%	7.81 ± 0.69^{ab}	64.41 ± 4.97^{e}	6.12 ± 1.56^{c}
Alginate 0.04	10.07 ± 4.66^{a}	83.63 ± 1.73^{abc}	4.20 ± 0.18^{c}
Alginate 0.07	9.92 ± 1.15^{ab}	86.63 ± 1.18^{ab}	4.51 ± 0.22^{c}
Algnate 0.10	8.12 ± 3.20^{ab}	80.47 ± 3.19^{bcd}	4.85 ± 0.24^{c}

Value with different letters and the same column are significantly different at a = 0:05

The number of void or solid structure as shown by the SEM observation could lead to reduce tensile Strength (Kirdpondpattara *et al*., 2015), whereas Zhou *et al*., 2007 suggested the formation of BC ribbon with a wider size can affect the mechanical properties

Grande *et al*., (2008) also have same result that tensile strength decreased due to the addition of starch. Consequently, it affects the mechanical properties such as tensile strength, as also noted by other researchers. Decreasing tensile strength may be associated with the decrease of the crystallinity (Watanabe *et al.*,1994).

Water holding capacity: Water holding capacity demonstrate the ability of the structure of food to prevent / defend water out of the three-dimensional structure (Hermansson, 1986). Water retained on BC that form 3-dimensional network is an important property for application in the field of food and non-food (Jagannath *et al.,* 2008).

The effect of polysaccharides presence in the medium of bacterial incubation on the WHC is represented by table 2. The addition of starch to the medium able to increase WHC compared to medium without addition of polysaccharide, but WHC decrease with increasing the concentration of starch into medium. Similar results were shown by medium added alginate. The adding of agar to the medium showed the opposite result when compared to the starch and alginate. The Adding of agar to the medium resulted in decreasing WHC of BC and the WHC also decrease by increasing the concentration of agar into medium. The addition of starch to the medium resulted BC with WHC greater than agar and alginate.

WHC level is associated with structural arrangement of BC. The amount of water absorbed by the BC is strongly influenced by the density of a 3-dimensional matrix of BC. The more the empty space or porous cellulose, the more water that can enter and be absorbed in the matrix material (Islam *et al.*, 2012). Based on SEM observations showed a different structure forms. The addition of starch in medium produces cellulose to form a net that has many pores or empty space so that the WHC increase because much water entry and absorbed on cellulose while adding agar and alginate in the medium resulted in a layer of the ribbon-shaped wide and has little pores or empty spaces than starch.

A decreasing crystallinity of the BC from the medium which added starch and alginate resulting cellulose more amorph, many pores or a larger empty space.

A material having porous fibrils and high surface area high WHC properties (Dahman, 2009). Chang *et al*., (2012) also noted the larger number of amorphous regions accelerates and promotes water permeation into cellulose network that results in the larger WHC. These results were supported by the observation using SEM that showing the addition of polysaccharides were able to form new matrix with different denser levels. The addition of agar into medium resulted in a decrease in WHC since formed a ribbon or layer that covers the gaps that exist in the BC matrix, as shown in observation using SEM.

The results also showed an increasing in the concentration of the three polysaccharides result in decreasing WHC. Similar results were also obtained by Islam *et al.,* (2012), which indicates the greater concentration of SSGO is added to the medium, its WHC decreases.

Rehidration ratio: The adding of starch to the medium produce BC with rehydration ratio was greater than the medium without adding of polysaccharide, and the ratio rehidration more greater with increasing the concentration of starch into medium. Similar results were shown by agar and alginate, especially when the concentration of agar and alginate increase into medium. BC produced using starch gave rehydration ratio greater than agar and alginate. The adding of starch at a concentration of 2% give greatest rehydration ratio (13, 30%).

Rehydration ratio is the ratio of the amount of water that can be absorbed by the BC after drying with the amount of water in the wet cellulose. The effect of the addition of polysaccharides on the Rehydration ratio is presented in table 2. It is seen in the table that rehydration ratio increases with the presence of starch, agar, and alginate. The highest effect is shown by starch. Furthermore, the increasing concentration polysaccaride result in the larger rehydration ratio.

Increasing rehydration have assosiated with degree crystallinity of BC (Lin *et al.*, 2009). The adding of starch into medium showed greater changes than other polysaccharides because the resulted matrix more porous(loose network) than agar and alginate. Three dimensional network of BC that have many pores will increase the space for the water to get into the BC matrix (Huang, *et al.*, 2010; Lin *et al*., 2009; Chang *et al*., 2012).

This results supported by the observation using SEM that showing the addition polysaccharide able to form new matrix with different denser levels.

Conclusions

The adding polysaccaride into the medium can decrease crystallinity from BC. The decrease crystallinity impact on the change in the 3-dimensional structure of BC and subsequent effect on other physical properties. The adding of starches, agar and alginate resulted in decreased tensile strength, increasing WHC and rehydration ratio. Addition of starch gave more pronounced effects than that of agar or alginate. The addition of starch in the medium resulted in decreasing the crystallinity and changes the physical properties greater than agar and alginate

Acknowledgements

This work was financed by Ministry of Research Technology and Higher Education (Kemenristekdikti).

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