Incorporation of pectin during biosynthesis of bacterial cellulose by *Gluconacetobacter xylinus* InaCC B404: Possibility for producing green food packaging

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[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada. Jl. Sekip Utara, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia
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Abstract. *Agustin S, Wahyuni ET, Suparmo, Supriyadi, Cahyanto MN. 2021. Incorporation of pectin during biosynthesis of bacterial cellulose by* Gluconacetobacter xylinus *InaCC B404: Possibility for producing green food packaging. Biodiversitas 22: 2548-2553.* Bacterial cellulose-pectin biocomposite is developed as a potential environmentally friendly food packaging material. The objective of this research was to investigate the effects of pectin supplementation to the growth medium of *Gluconacetobacter xylinus* on the properties of the biocomposite produced. Pectin was added into the growth medium at different concentrations, i.e.: 0.1, 0.2 and 0.3% (w/v). The bacterial cellulose-pectin biocomposites produced were analyzed for structure, mechanical strength, crystallinity, morphology, color and opacity. FTIR analysis suggested that interaction between bacterial cellulose and pectin was hydrogen bonding. Generally, pectin addition decreased the crystallinity, tensile strength, tear strength and lightness of biocomposite produced. The tensile and tear strength of bacterial cellulose-pectin biocomposites were in the range of 23.05-25.72 MPa and 13.30-15.72 g/μm respectively, comparable to those conventional non-biodegradable plastics such as high-density polyethylene and low-density polyethylene with opacity much lower than HDPE. These results suggesting the possibility of cellulose-pectin biocomposite as semi-translucent green food packaging.

Keywords: Bacterial cellulose, *Gluconacetobacter xylinus*, pectin

INTRODUCTION

Cellulose is the foremost inexhaustible renewable polymer in nature, comprising glucose units connected by β-(1,4) bonds, created by different life forms such as plants, bacteria, algae, and animals. Bacterial cellulose (BC) is an exopolysaccharide formed primarily by the bacteria *Gluconacetobacter xylinus*. Bacterial cellulose possesses a chemical structure similar to that of plant cellulose, with several advantages including reticulate structure stabilized by inter-and intra-hydrogen bonds, high purity, high degree of polymerization (up to 8000) and high mechanical strength (Barud et al. 2011). Despite these advantages, the high degree of crystallinity possessed by bacterial cellulose makes this material has the disadvantage of being very stiff, thus limiting its application as packaging material. Bacterial cellulose, on the other hand, has abundant hydroxyl groups on the surface, so that modification of the cellulose structure is possible (Hu et al. 2014).

By controlling the conditions of cultivation, the shape and structure of bacterial cellulose can be regulated. Several studies have shown that adding additives to bacterial growth media can modify the composition, morphology and characteristics of the bacterial cellulose produced. These compounds can infiltrate the cellulose structure in the biosynthesis process, or can also influence the formation of fibrils or ribbons, thereby changing the structure of the resulting cellulose, including changing the size of the fibrils, degree of crystallinity and crystal size (Klemm et al. 2006; Ruka et al. 2013). Bacterial cellulose is composed of nanometer-sized subfibrils that are crystallized into microfibrils (Yamanaka et al. 2000). Additives in the cellulose-producing bacteria growth media will interfere with the crystallization of glucan chains (Tokoh et al. 2002), thus affected the characteristics of cellulose produced.

Some studies have been done to evaluate the effect of additives incorporated in the growth medium of cellulose-producing bacteria. The fuse of water-dissolved polymers such as carboxymethylcellulose (CMC), hydroxypropylmethyl cellulose (HPMC) and methylcellulose (MC) into the growth medium causes a decrease in crystallinity and crystallite size, as well as increases heat steadiness, pore size, capability of water retention and capacity for ion absorption (Chen et al. 2011). The addition of Na-alginate to the medium resulted in macromorphological changes in cellulose, reducing crystallinity and crystallite size (Zhou et al. 2007; Sya'di et al. 2016). Some additives that have been tested for their impact on the yield, morphology and crystalline structure of bacterial cellulose are chitosan,

starch, agar, gelatin, pectin, poly-3-hydroxybutyrate, aloe vera and xyloglucan (Saibuatong and Phisalaphong 2010; Ruka et al 2013; Chen et al. 2014; Jia et al. 2017; Szymanska-Chargot et al. 2017; Sya'di et al. 2017a).

Pectin, a complex polymer of heterosaccharide found in almost all higher plants in the middle lamella or the primary cell wall, is an ester of methylated D-galacturonic acid. In plant cells, pectin forms independent network even though it is close to cellulose (Tokoh et al. 2002). Several studies have used pectin as an additive in growth media for cellulose-producing bacteria to understand the interactions of the two polysaccharides in plant cell walls (Lin et al. 2018; Lin et al. 2016; Szymanska-Chargot et al. 2017). However, research on bacterial cellulose-pectin (BCP) biocomposite as an environmentally friendly food packaging material has not been widely explored. Increased concern about environmental pollution and environmental imbalances as a result of the widespread use of non-biodegradable plastic packaging materials has stimulated research into alternative food packaging materials based on biopolymers, including bacterial cellulose and pectin. This research aimed to investigate the physicochemical properties of bacterial cellulose modified in situ with the addition of pectin to the growth medium of *G. xylinus* InaCC B404 and associate it with the possibility of making the biocomposite as green packaging material.

MATERIALS AND METHODS

Materials
The microorganism used in the production of biocomposites was *Gluconacetobacter xylinus* InaCC B404 obtained from the culture collection of the LIPI Biotechnology Center Bogor, Indonesia. The strain was cultured in Hestrin-Schramm (HS) media containing 2% D-glucose, 0.5% peptone, 0.5% yeast extract, 0.27% Na_2HPO_4 and 0.115% citric acid monohydrate.

Methods
Preparation of bacterial cellulose composite
Bacterial cellulose was produced by *G. xylinus* InaCC B404 using Hestrin Schramm media as described by Sya'di et al. (2017b). Various concentrations of pectin (0.1, 0.2 and 0.3% w/v) were incorporated into the media. Cultures were grown at 30°C for 7 days under static conditions. After the incubation period was completed, the pellicle formed was harvested and boiled in 0.1 N sodium hydroxide for 30 minutes accompanied by rinsing with pure H_2O until neutral pH was achieved. For the purposes of measuring morphological, physical, and mechanical properties of the composite, the harvested pellicles were then dried by freeze-drying method for 15-24 hours.

Physicochemical analysis
BC morphology
Scanning electron microscope instrument of SEM JEOL JSM-6510 LA type has been used to conform the morphology of the BC and BCP composites according to Ul-Islam et al. (2011). Sputter was coated with gold in freeze-dried samples. Acceleration voltage at 15 kV was used for sample examination.

BC crystallinity
Using a Rigaku Miniflex600, freeze-dried samples of BCP composites were X-rayed. X-ray diffraction patterns (40 kV, 15 mA) were reported at the CuKα radiation wavelength (λ = 1.54°A) based on the methodology of Huang et al. (2010). Samples were scanned at scan speed of 0.02° min⁻¹ from 10-30° 2θ-range. The crystallinity (Cr) and crystallite size (CrS) were determined depended on X-beam diffraction estimations. Crystallinity was determined using equation as follows:

$$Cr\ (\%) = \frac{Sc}{St}\ x\ 100\%$$

Where: Sc is sum of crystalline area and St is sum of total area. Scherrer equation was used to obtained CrS as follows:

$$CrS = \frac{K\lambda}{\beta cos\theta}$$

Where: K is the shape factor (0.9), λ is the x-ray wavelength (1.54°A), β is the full width at half maximum height (FWHM), and θ is the Bragg's angle (°).

Functional group
With a Shimadzu-8201 PC Corp. (Japan) spectrophotometer, FT-IR spectra were obtained with a resolution of 4 cm⁻¹, working in the range of 400-4000 cm⁻¹. All samples were mixed with KBr to produce KBr-pellet prior to spectra measurement (Ul-Islam et al. 2011).

Color and opacity
A colorimeter (CR-400 Konica Minolta, Japan) along with a CIELab color space was measured for film color. Values of L* (lightness: 0 = black, 100 = white), a* (-a* to greenness, +a* to redness) and b* (-b* to blueness, +b* to yellowness) were determined according to Wang et al. (2018). The total color difference (ΔE) was obtained using the following equation:

$$\Delta E = \sqrt{(\Delta L *)^2 + (\Delta a *)^2 + (\Delta b *)^2}$$

Where: ΔL*, Δa*, and Δb* are the differences between the color values of the pure BC film and the BCP films.

The opacity was reported according to the methodology of Wang et al (2018). The films were shaped into rectangular and positioned using air as a reference in the spectrophotometer test cell. Film opacity at 600 nm was reported as follows:

$$Opacity = {A_{600}}/{x}$$

Where: A_{600} is the absorbance at 600 nm and X is the film thickness (mm).

Mechanical properties

The tensile and tear strength of the film were measured using Universal Testing Machine (UTM) ZWICK Z.05-type mechanical tester based on ASTM-D882-02 (2002). The sample films were cut into strips of 2 cm width and 10 cm long prior to measurement. The thickness of all films was determined using a thickness gauge (Mitutoyo 7321) and then inserted into the locking grip. The films were extended with 50 mm of beginning hold detachment and 10 mm/min test speed.

Data analysis

In order to assess any important variations between the treatments, the data collected was further analyzed using Analysis of Variance (ANOVA) followed by Duncan's multiple range test at 95% confidence level (p≤0.05) by SPSS Statistic Software Version 25 (2017). All data were presented in the form of mean ± SD.

RESULTS AND DISCUSSION

Functional groups

Because of the strong bonding with hydrogen between cellulose molecules, the FTIR spectrum of BC (Fig 1.) showed a peak area of 3426 cm^{-1} suggesting stretching vibration of OH groups. The peaks in the areas of 2916 cm^{-1} and 1427 cm^{-1} are the stretching vibration region of the C-H groups due to symmetrical CH deformation (Ul-Islam et al. 2011). The area of deformational vibrations of OH groups of bound water is the peak indicated at 1642 cm^{-1}. The peaks in the 1500-1200 cm^{-1} range are sensitive to changes in chemical and molecular structures, while the peaks in the 1200-1000 cm^{-1} range correspond to the vibrations of the C-O-C and C-O ranges, and the peaks in the 895 cm^{-1} range indicate vibrations of the β-1,4 bonds (Atykyan et al. 2020).
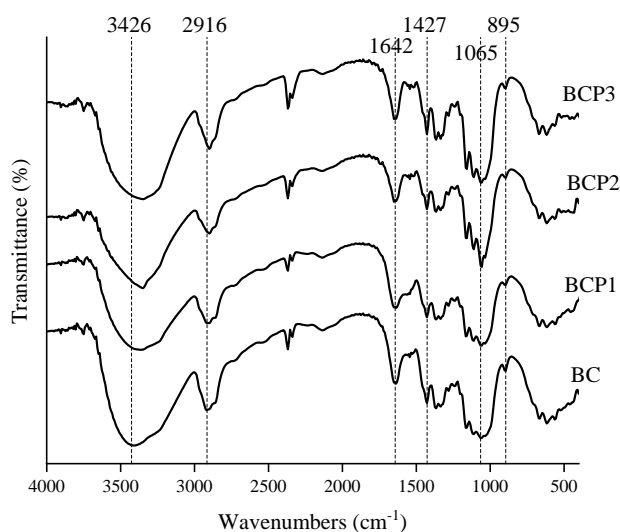
Compared to BC native, FTIR spectra of BCP revealed few changes such as decreased intensity and shifting of wavenumbers. The peaks in the 2916 cm^{-1} area were seemed to be sharpened and narrow with pectin addition, indicating the appearance of amorphous region in cellulose structure (Rathinamoorthy et al. 2019). Another peak change appeared in the region of 1065 cm^{-1} which could be owed to changes in C-O-C and C-O vibration stretching. This result suggests that there are interactions between BC and pectin incorporated in the structure of BC, certainly because of the creation of an interconnected network between two polymers of similar structure and chemical features (Cacicedo et al. 2018).

Mechanical properties

The addition of pectin to the *G. xylinus* growth medium resulting in biocomposites with lower mechanical strength than native BC (Table 1). This finding is in line with that obtained by Szymanska-Chargot et al. (2017) and Gu and Catchmark (2014). The type of polysaccharide added to the *G. xylinus* growth medium greatly influenced the interaction and structure of the BC fiber formed. The decreased tensile and tear strength of BCP biocomposites was due to increasing amorphous region in the BCP structure as proven by decreasing crystallinity as observed by XRD analysis and FTIR spectra. Reduced tensile strength resulted in increased deformability as suggested by Baron et al. (2017), which also means reduced stiffness of biocomposites produced.

The tensile and tear strength of all biocomposites were comparable to those conventional non-biodegradable packaging materials such as high-density polyethylene (HDPE) and low-density polyethylene (LDPE) which have tensile strength values of 17.3-44.8 and 8.2-31.4 MPa, and tear strength values of 0.80-2.40 and 8-12 g/μm, respectively (Selke et al. 2015).

Figure 1. FTIR spectrum of BC incorporated with various pectin concentration
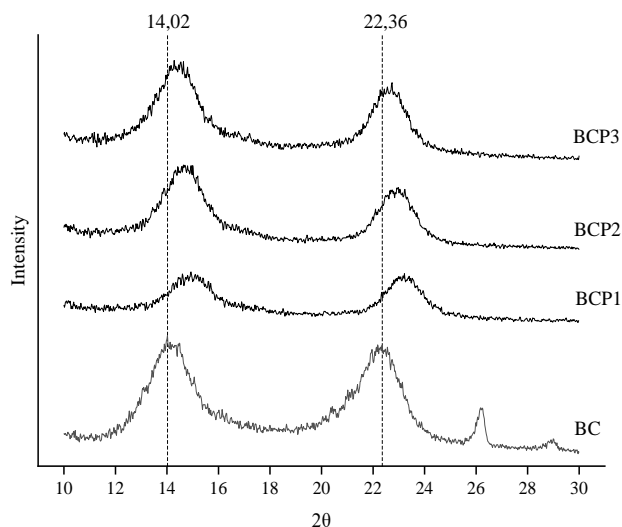
Figure 2. XRD pattern of BC incorporated with various pectin concentration

Table 1. Crystallinity and mechanical properties of BC incorporated with various pectin concentrations

Sample	Crystallinity (%)	Crystallite size (nm)	d-spacing		Tensile strength (MPa)	Tear strength (g/µm)
			110	002		
BC	90.63	3.06	0.3147	0.2036	108.59±0.53b	27.77±0.50b
0.1% BCP	77.56	3.41	0.3047	0.1981	23.05±0.55a	12.80±0.99a
0.2% BCP	79.19	2.68	0.3158	0.2047	25.72±0.95a	9.63±0.61a
0.3% BCP	79.62	1.81	0.3134	0.1975	24.16±0.51a	15.72±0.80a

Table 2. Color properties of BC incorporated with various pectin concentrations

Sample	L*	a*	b*	Color Difference	Opacity
BC	70.45±0.01c	-3.23±0.01d	-0.98±0.01b	-	6.32±0.03a
BCP 0.1%	37.40±0.16a	-1.70±0.03a	-2.25±0.02a	43.28±1.19	6.57±0.94a
BCP 0.2%	37.73±0.00b	-2.12±0.01c	-1.44±0.01d	40.71±1.20	7.02±0.11a
BCP 0.3%	37.71±0.01b	-1.91±0.02b	-0.32±0.02c	41.82±1.21	21.79±0.57b
HDPE*	93.70±0.10	-0.61±0.02	3.20±0.10	-	35.20±0.20

Note: *Bonilla et al. (2020)

Crystallinity properties

Some crucial characteristics to determine the behavior of crystalline material are observed using X-ray diffraction. BC's general XRD spectrum, which is often accompanied by other relatively weak peaks at ~16°, are the two intensive peaks at $2\theta = \sim14°$ and $\sim22°$ which are generally identified as typical cellulose I profile (Sugiyama et al. 1991).

The diffractogram of bacterial cellulose produced with pectin addition was analogous to that of native BC, but with slightly different peak width and intensity. Figure 2 showed that the XRD pattern of BC revealed two specific peaks at $2\theta = 14.02°$ and $22.36°$. The addition of pectin shifts the 2θ peak and decreases the intensity, while the peak pattern remains the same. This indicates that cellulose crystalline structure was not affected by the pectin supplementation. However, addition of pectin resulted in decreased crystallinity of BCP compared to native BC. This may be due to intermolecular reaction between pectin and cellulose, which restricted the movement of BC molecular chains (Cai et al. 2016). Another possible reason is the formation of amorphous BC or other amorphous polymers (Dayal and Catchmark 2016). The BCP biocomposite also showed changes in crystal size and d-spacing (Table 1). The incorporation of pectin reduced the crystal size and improved the d-spacing value, indicating the formation of a wider amorphous area in the resulting biocomposite, thus decreasing crystallinity. Pectin can interfere with the crystallization process during the formation of BC microfibrils, resulting in a biocomposite with lower crystallinity (Dayal and Catchmark 2016).
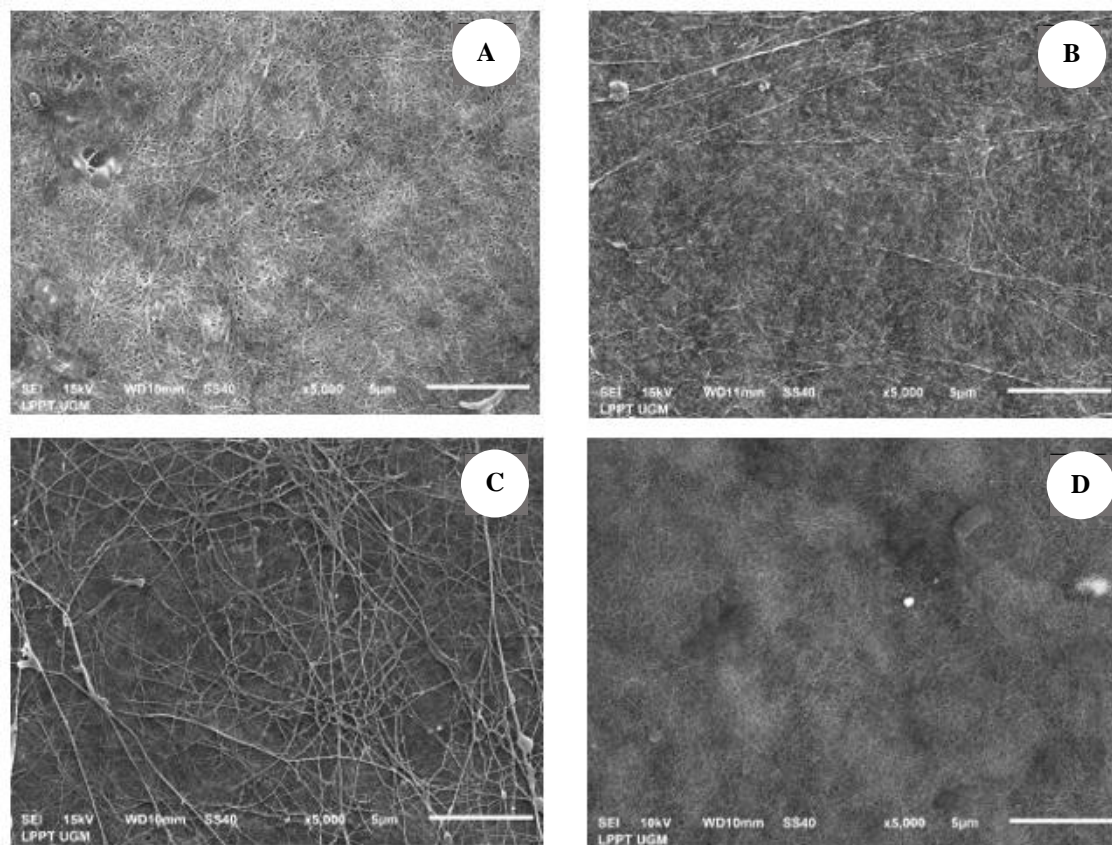
BC morphology

BC biosynthesis is well known for its' single-directional growth and crystal formation, in which glucose molecules are bound together with β (1→4) glycosidic bond (Tokoh et al. 2002). From Figure 3a the morphology of BC native can be seen as being some interwoven cellulose fibrils with many hollow parts. The arrangement of these fibrils forms pores with different diameter sizes (Lin et al. 2018). The presence of pectin impacted the size of the pore formed, in which the pectin appeared to fill the empty space between the fibrils, resulting in a composite with a smaller pore size (Figure 3b). Pectin was also seen to form a layer on the cellulose surface (Figure 3c), or agglomerate around cellulosic fibrils due to the non-uniform dispersion of pectin (Figure 3d). It was validated, as alluded to in the FTIR study, that pectin may render the cellulose fibril intermolecular bond.

Color properties and opacity

CIELab color values and opacity measurements of biocomposites as affected by pectin addition are presented in Table 2. The native BC film had the highest L* (lightness) value, which indicated that this film was more white and clear compared to other biocomposites with pectin addition. Presence of pectin in the biocomposites had a direct influence on the color and opacity of the films. This is due to the natural appearance of pectin granule which showed a light brownish color. Incorporation of pectin in the BC structure resulting in lower L* value with higher redness (a*) and lower yellowness (b*). There are no significant differences observed in color difference for all BCP treatments, suggesting no visual difference between BCP films. As shown in Table 2, the opacity value of biocomposite was increased by adding pectin concentration but with no significant differences, except for 0.3% BCP. Higher value of opacity indicating lower transparency (Atef et al. 2014). BCP showed a lower opacity compared to conventional HDPE plastic, which indicating the potential application of BCP as transparent biodegradable food packaging material.

Figure 3. Surface morphology of: A. BC, B. 0.1% BCP, C. 0.2% BCP, D. 0.3% BCP

In conclusion, incorporation of pectin into BC matrix generally decreased tensile and tear strength, crystallinity properties, lightness and opacity of the biocomposite generated by interlinkage of pectin and BC polymer through H-bonding. Pectin can spread well in the BC matrix, resulting in better film quality based on tensile strength and opacity values. The biocomposite was slightly transparent with excellent mechanical properties compared to conventional non-biodegradable plastics, suggesting the potential of BCP as a green packaging alternative for food products.

ACKNOWLEDGEMENTS

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