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Effects of *Gluconacetobacter xylinus* Concentrations on the Physicochemical Properties of Bacterial Cellulose from Coconut Water Medium

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ABSTRACT

Utilization of coconut water into a bacterial cellulose product is promising to take advantage of the abundant natural resources. The synthesis of bacterial cellulose using coconut water media requires optimal conditions to achieve the best characteristics as a bioplastic component. This study aims to determine the effect of variations in the concentration of Gluconacetobacter xylinus starter. The type of research is experimental using a one-factor Complete Random Design (RAL) by going through a fermentation process influenced by variations in starter concentrations (5%, 7%, 9%, 11%, 13%, 15%) with test parameters including water vapor permeability, tensile strength, and crystallinity index. The results showed a significant influence ($p \leq 0.05$) of water vapor permeability testing, tensile strength testing, and yield testing. The crystallinity index test of the best treatment obtained a concentration of 15%. The best treatment of 15% resulted in the average water vapor permeability (3.84×10^{-7} g/(m^2/day)), tensile strength (69.25 MPa), crystallinity index (91.35%), and yield (75.70%). Bacterial cellulose produced using a 15% starter can be used as bioplastic packaging material and allows for further research by combining it with other materials to obtain the best and most affordable bioplastic formulation.

1. INTRODUCTION

Coconut is one of Indonesia's most abundant natural resources. Coconut water can be explored to produce bacterial cellulose products; one of them is nata de coco. Bacterial cellulose has even become a product in the industry with well-known brands. Bacterial cellulose or "nata" is one of the processed types of organic food products with a fairly high fiber content with a fermentation process. It is assisted by the bacteria *Gluconacetobacter xylinus* (Putri *et al.*, 2021). Bacterial cellulose is formed by millions of cellulose planted in coconut solution, producing many strands of cellulose threads, forming a gel-like texture.

Using food packaging with plastic products is a cheap and effective alternative. However, Petroleum-derived plastics besides polluting water and soil, are also responsible for various problems due to the incineration of plastic waste. Burning synthetic plastics produces tremendous carbon dioxide and affects global warming (Gupta *et al*., 2022). One innovation that can be done is bioplastics. Packaging is made of renewable polymers and consists of organic monomers in starch, cellulose, proteins, lipids, and microorganisms called bioplastics (Mashuni *et al.,* 2021).

Bacterial cellulose, which consists of millions of cellulose fibers, can be used as one of the materials in the manufacture of bioplastics. According to Elfiana *et al.* (2018), bacterial cellulose can absorb water so that it degrades faster, due to the properties of nata which have the main component, namely cellulose polymer. Cellulose polymers produced by agriculture have an innate character, namely thermoplastic which has the potential to be used as bioplastics.

Research on bioplastics using BC showed good packaging characteristics but is not economical because it still uses expensive materials. Almost all research uses a defined medium named Hestrin and Schramm medium, which has a composition of glucose, peptone, yeast extract, disodium phosphate, and citric acid and is adjusted to pH 6 (Cazon & Vazquez, 2021). Thus, one of the major challenges of fermentation processes is to find a new low-cost medium such food processing waste (molasses, waste form fruit processing and coconut water) (Fernandes *et al.*, 2020; Cazon & Vazquez, 2021). Among these materials, coconut water has been used for commercial production (widely used as nata de coco). Coconut water is a suitable medium for bacterial cellulose biosynthesis because it naturally contains simple sugars, minerals, vitamins, and others. The production of nata can be influenced by external and internal factors, such as carbon sources, nitrogen sources, fermentation temperatures, medium acidity levels,fermentation time, and starter concentrations of *Gluconacetobacter xylinus* (Ismaya *et al*., 2021).

The change of medium, fermentation time, and starter concentrations of *Gluconacetobacter xylinus* influences the formation of fibrils or ribbons, thereby changing the structure of the resulting cellulose, including the degree of crystallinity (Agustin *et al*., 2021; Pham & Tran, 2023) and further influencing the physical and mechanical properties (tensile strength, elongation, Young's Modulus) (Sya'di *et al*., 2017; Adriani *et al*., 2020). This study aims to determine the effect of starter concentration *Gluconacetobacter xylinus* on the characterization of bacterial cellulose. Bacterial cellulose produced by *G. xylinus* from coconut water media at the optimal concentration has characteristics that can be used as bioplastic material.

2. MATERIALS AND METHODS

2.1. Materials

The ingredients used in the treatment of coconut water into bacterial cellulose, namely coconut water derived from old fragrant pandan coconuts obtained from the Pedurungan market, Semarang, Central Java. Sucrose, ammonium sulfate, acetic acid, and *Gluconacetobacter xylinus* starter obtained through the National Research and Innovation Agency.

2.2. Bacterial Cellulose Preparation

The production of bacterial cellulose was carried out with a coconut water medium composition with varying concentrations of *G. xylinus* starter (Table 1). Coconut water heated to 90°C then added 5% sucrose and ammonium sulfate food grade 0.5%. After coconut water reaches a temperature of 40°C – 48ºC, 1% acetic acid was added until the resulting pH ranges from of 4.5–4.7. The coconut water was sterilized using an autoclave to a temperature of 121°C for 15 minutes. Bacterial inoculum was carried out after the sterilization process is completed, after the tempetarure medium reach 30°C, the bacterial inoculum adjusts with variation concentration (5–15%). After that, the coconut water solution was fermented for 7 days at a temperature of 30°C in an incubator. To neutralize the growth rate of bacterial cellulose, boiling at 100°C was carried out for 15 minutes (Kurniawati & Dzakiy, 2015).

Table 1. Medium composition and starter concentration

Materials	Unit	Treatment					
		P1	P2	P3	P4	P5	P6
Coconut water	mL	500	500	500	500	500	500
Starter	%	5	7	9	11	13	15
Glucose	%	5	5	5	5	5	5
ZA (Zwavelzure Ammoniak)	%	0.5	0.5	0.5	0.5	0.5	0.5
Asetic acid	%	1	1	1	1	1	1

2.3. Design of Experiments

This study used experimental design namely a one factorial Completely Randomized Design (CRD), with varying concentrations of *Gluconacetobacter xylinus* starter with 6 treatment starter concentrations (5%, 7%, 9%, 11%, 13%, 15%). In determining the number of repetitions, the Federer formula was used, which is $(t-1)(r-1) \geq 15$, resulting in 4 repetitions and 6 treatments, so the total number of samples is 24 experiments.

2.4. Parameters and Measurements

2.4.1. Water Vapor Permeability (WVP)

The method refers to Agustin *et al.* (2021), which is gravimetry in accordance with ASTM E96 (American Standard Testing and Material). The test tube was filled by a quarter of its volume and then closed with the cut sample. The container is weighed and placed in a dry cabinet with a specific temperature and humidity. Then the weight of the container caused by the reduction of moisture was determined by weighing it at intervals of 30 minutes and carried out for 2.5 h. Water vapor permeability (WVP) was calculated as the following:

$$\mathbf{WVP} = \frac{WVTR \times t}{A \times P\ (R_1 - R_2)} \quad (1)$$

where t is film thickness (m), WVTR is Water Vapor Transmission Rate (g.m^{-2}.h^{-1}), A is film area (m^2), P is saturated water vapor pressure (Pa), and R_1-R_2 is RH difference inside and outside the container.

2.4.2. Tensile Strength

Testing refers to Hendrawati *et al.* (2019), using samples customized to ASTM D-638 standards (American Standard Testing and Material). The Brookfield CT3 tool was used to read the tensile strength value. The test was carried out with the help of a testing machine by hooking both ends of the sample on the machine. Maximum adjustable distance, speed on loading, and Range load or force. The sample was pulled slowly until the sample is disconnected.

$$\mathbf{s} = \frac{F_{max}}{A} \quad (2)$$

where σ is tensile strength (N/mm^2), F_{max} is maximum force (N), and A is surface area (mm^2)

2.4.3. Best Concentration Treatment

The best treatment of bacterial cellulose characteristics based on the concentration of *Gluconacetobacter xylinus* starter was obtained from the highest ranking using the Composite Performance Index (CPI) method with the determination of weights on the parameters of the second test. The weight determined in the water vapor permeability test parameters was 60%, and the tensile strength test was 40%.

2.4.4. Crystallinity Index

The crystallinity index (XCR) was calculated based on the following equation Setiawati *et al*., (2015). Determination of the crystal degree value used Shimadzu X-ray Diffraction-7000.

$$\mathbf{XCR} = \frac{I_{200} - I_{am}}{I_{am}} \quad (3)$$

where *XCR* is sample crystallinity, I_{200} is density of the cellulose crystal part (2Θ = 22.8°), and I_{am} is intensity of amorphous part of cellulose (2Θ = 18°).

2.4.5. Yield

Determination of yield refers to (Marlinda & Hartati, 2019). The resulting bacterial cellulose was first neutralized and washed using aquades then drained for 30 minutes. To find out the weight, it was weighed and calculated using the following equation:

$$\mathbf{Yield\ (\%)} = \frac{Weight\ produced}{Medium\ weight} \times 100\% \quad (4)$$

2.5. Data Analysis

The data obtained from the water vapor permeability test and tensile strength test were analyzed using ANOVA (Analysis of Variance), and the differences were further tested using the Duncan Multiple Range Test (DMRT). The best treatment for bacterial cellulose characteristics based on the WVP and tensile strength tests was determined using the Composite

Performance Index (CPI) method. The calculation steps included: (1) normalization of the matrix from the average of each treatment in both tests; and (2) the multiplication of the weight with the average results of each treatment in both tests. Then, the multiplication values from both tests were summed for each treatment. The total values of both are ranked to determine the best treatment.

3. RESULTS AND DISCUSSION

In this study, bacterial cellulose was produced by *G. xylinus* using coconut water media (Figure 1), and its characteristics were observed, including WVP, tensile strength, and crystallinity index.

Figure 1. Bacterial cellulose (nata) from coconut water

3.1. Water Vapor Permeability

Permeability testing is an critical factor because the material is tested for the moisture it passes through. WVP is one of the important parameters of food packaging because bacterial cellulose is used as a food packaging material (Yanti *et al.*, 2021). The enhanced water vapor barrier is advantageous for most food packaging applications, since water transfer through a packaging film may result in changes in food. The use of cellulose as reinforcement material elongates the water transmission path thus reducing the WVP texture and even promote microbial growth (Papadaki *et al*., 2022).

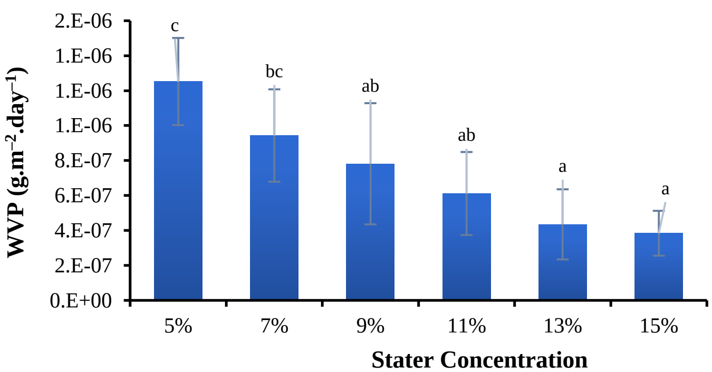

Figure 2. Effect of starter concentration on the average WVP of the resulted bacterial cellulose

The results of the permeability test analysis shown in Figure 2 show that the permeability of bacterial cellulose membrane cells decreases with each treatment. The analysis of the ANOVA statistical test showed that the treatment of starter concentration variations significantly affected the characteristics of cellulose bacteria WVP with ($p \leq 0.05$). The results were further tested with Duncan, who obtained a difference in the 5% treatment, which was different from the 9-15% treatment, and the 5% treatment was no different from the 7% treatment. The lowest average permeability value was produced at a concentration of 15%, 3.84×10^{-7} g/(m^2/day), while the highest was at a concentration of 5%, namely 1.26×10^{-6} g/(m^2/day).

Difficult water vapor penetration due to the strong structure of the crystal bond arrangement between the film constituent materials (Sari *et al.*, 2024). An increase in starter concentration decreases the value of WVP because the interaction between hydrogen bonds decreases the amount of hydroxyl available to bind water vapor. In addition, the strong bonds between hydrogen can block the rate of water vapor migration from the outside environment into the packaging (Wang *et al.*, 2018). If the formation of bacterial cellulose is not optimal, it causes the formation of hydrogen bond structures between fibers to be less than optimal, thus creating pores between fibers so that the value of permeability high yield (Boby *et al*., 2021).

3.2. Tensile Strength

The tensile strength test parameters help to determine the flexibility and flexibility of the film. Good mechanical properties allow the designation of biomaterials as bioplastics (Yanti *et al.*, 2021). The analysis of the tensile strength test in Figure 3 showed that the tensile strength in bacterial cellulose membrane cells tends to increased but at starter concentration 15% decreased. The test results showed that the concentration of 13% had the most significant tensile strength value, 71.36 MPa, while the one with the lowest tensile strength value was at a concentration of 5% of 14.935 MPa. The analysis of the ANOVA statistical test showed that the treatment of starter concentration variation significantly affected the tensile strength characteristics of bacterial cellulose with ($p \leq 0.05$). The results of the further test were obtained that the difference in the treatment of 13%, 15% had a difference with the treatment of 5%, 7%, while the treatment of 7%, 9%, 11% had similarities.

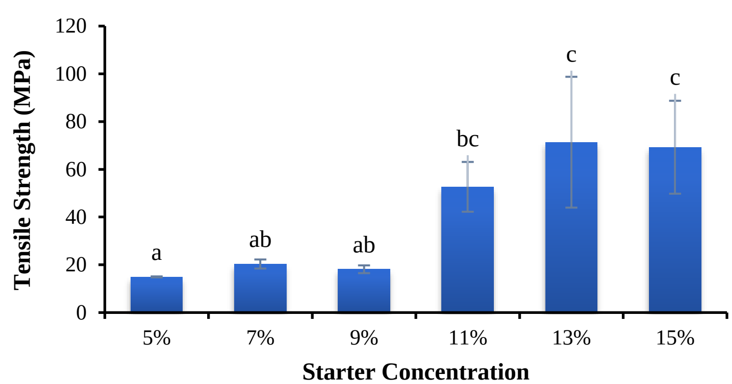

Figure 3. Effect of starter concentration on the average tensile strength of the resulted bacterial cellulose

The structure formed in bacterial cellulose is due to the mixture of making materials and the concentration of the starter given. The high tensile strength value is produced because the cavities between the matrices tightly closed by the surface layer are getting smaller. Tensile strength is related to crystallinity, if the tensile strength value is high, the crystallinity yield will also be high, because elongation at break tends to be low in crystal structures with regular and dense fibrils (Sya'di *et al.*, 2017).

According to Wang *et al.,* (2018), the bacterial cellulose scale with the addition of a high starter causes the interaction of hydrogen bonds that are formed to be denser and more easily formed polymers. High concentration of bacterial cellulose limit the interaction between polymers in their space for movement. This is because the high interaction of hydrogen bonds makes the bacterial cellulose formed have a denser texture.

3.3. Best Concentration Treatment

Determination of the best treatment of bacterial cellulose characteristics with variations in the concentration of the use of *Gluconacetobacter xylinus* starter based on two tests (WVP and tensile strength). Based on Table 2, it shows that the treatment with a starter concentration of 15% is the best treatment produced so that it ranks highest in the calculation of the Composite Performance Index (CPI) method, with the sum of the results of the WVP test and tensile strength of 6.86×10^1.

Table 2. Best treatment

Concentration Treatment	Test Results				Total Weighting	Rank
	WVP Test	Result	Tensile Strength Test	Result		
5%	3.05×10^1	1.83×10^1	100.0	40.0	5.83×10^1	4
7%	4.70×10^1	2.82×10^1	73.45	29.38	5.76×10^1	5
9%	4.91×10^1	2.95×10^1	82.40	32.96	6.24×10^1	2
11%	6.28×10^1	3.77×10^1	28.37	11.35	4.90×10^1	6
13%	8.83×10^1	5.30×10^1	20.94	8.37	6.14×10^1	3
15%	1.0×10^2	6.0×10^1	21.57	8.63	6.86×10^1	1
Weight	60%			40%		

Note : (1) is the highest score, and (6) is the lowest score

3.4. Crystallinity Index

Diffraction analysis using X-rays aims to discover the X-ray pattern in the crystallinity of bacterial cellulose. The crystallinity index parameter shows the relationship between permeability and tensile strength parameters. Research by Sya'di *et al.* (2017), shows that the relationship between the crystallinity index WVP and the tensile strength is directly proportional. The decrease in tensile strength also aligns with the decrease in bacterial cellulose crystallinity (Agustin *et al.*, 2021).

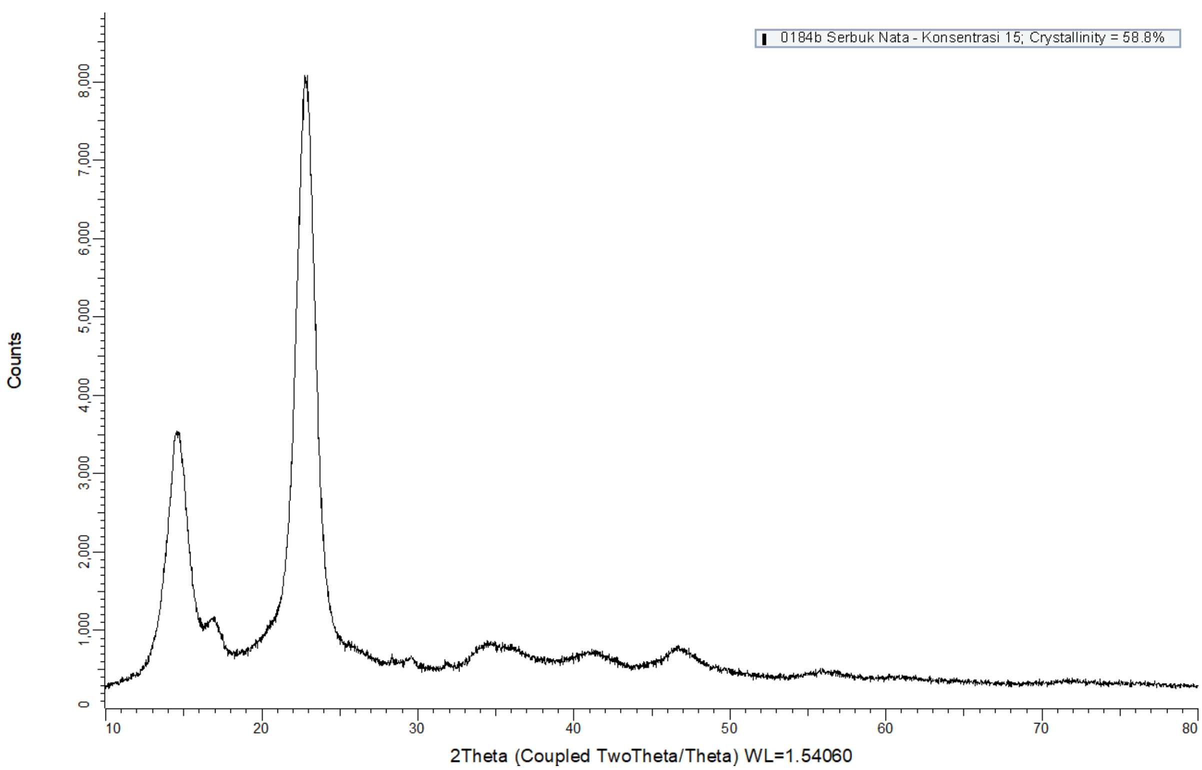

Figure 4. XRD analysis factograms produced at a concentration of 15%

Figure 4 shows the results of the bacterial cellulose diffractogram, the horizontal line explanation shows the magnitude of the diffraction angle and the vertical line shows the intensity of the reflected light. Calculation using the Segal *et al.*, (1959), at a concentration of 15% shows a percentage of crystallinity of 91.35% calculated from the level of light intensity reflected at an angle of 18° and 22.8°. The higher the concentration of the *Gluconacetobacter xylinus* starter given, the greater the percentage of crystallinity index produced, because the assembly of cellulose polymer chains produces crystalline nanofibers and the more bacteria are added, the denser the fibers are produced (Pham & Tran, 2023). The more starter used at the beginning, the greater the formation of hydrogen bonds, leading to a more organized structure and a wider crystalline area percentage. BC crystallinity is related to inter- and intramolecular hydrogen bonds formed during BC aggregation stages,starting from subfibrils, microfibrils to pellicles forming (Agustin *et al.*, 2021). The activity of bacteria significantly influences their crystallinity index because the bacteria secreted cellulose polymers that are used to assemble cellulose polymer chains and produce crystalline fibers (Suryanto, 2017). An increase in the number of bacteria in cellulose has the potential to increase the crystallinity value, which also affects the characteristics of tensile strength and WVP.

3.5. Yield

From the results of the analysis in Figure 5, it can be seen that the number of starters added in the bacterial cellulose manufacturing medium has an influence on the yield. Factors that affect nata yield include fermentation duration, the addition of ingredients (sugar, vinegar, and urea), the use of hollow lids, and the use of sterilized equipment. The use of different microorganisms also affects the fermentation results in terms of sensory, physical, and chemical properties (Putri *et al*., 2021). The results in Figure 5 show that the average value of bacterial cellulose yield fluctuates. The 5% starter variation give the highest yield, 75.70%. The analysis of the ANOVA statistical test showed that the treatment of starter concentration variation significantly affected yield of bacterial cellulose with ($p \leq 0.05$). The further test results showed that the difference in the treatment of 11% differed from the treatment of 5%, 7%, 9%, 13%, and 15%.

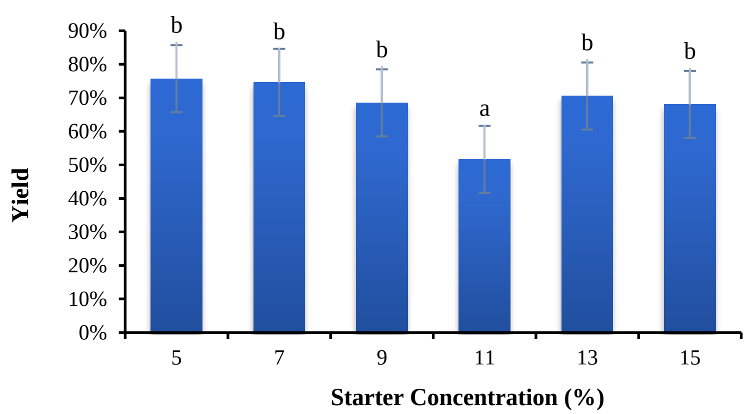

Figure 5. Effect of starter concentration on the average yield of bacterial cellulose

The more cellulose is formed, the thicker the nata produced, and will be directly proportional to the yield produced. Yield is affected by substrate variations, material composition, environmental conditions and capabilities of *Gluconccetobacter xylinus* in producing cellulose (Najri *et al*., 2022). The addition of starters with higher concentrations without the addition of nutrients results in bacterial activity in forming cellulose to be inhibited by bacteria *Gluconacetobacter xylinus* Because the energy needed for its formation is less and with the addition of a certain amount of starter can break down extracellular sucrose into glucose and fructose as a source of cell metabolism which is used as energy for the formation of cellulose (Marlinda & Hartati, 2019).

Bacterial cellulose produced using a 15% *G. xylinus* starter and coconut water media can be used as bioplastic packaging material because it exhibits good physicochemical properties, a high yield, and allows for further research by combining it with other materials to obtain the best and most affordable bioplastic formulation.

4. CONCLUSION

The variation in the concentration of *Gluconacetobacter xylinus* starter had a significant effect ($p \leq 0.05$) on water vapor permeability, tensile strength and yield. The crystallinity index based on the best treatment 15% (WVP3.8 x 10^{-7} g/(m^2/day), tensile strength 69.25 MPa) is 91.35%. Bacterial cellulose produced using coconut water media can be used as bioplastic packaging material, and allows for further research by combining it with other materials to obtain the best and most affordable bioplastic formulation.

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