Characteristics of bacterial cellulose derived from two nitrogen sources: ammonium sulphate and yeast extract as an indicator of smart packaging on fresh meat

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Abstract. Intelligent packaging equipped by an indicator to detect changes of food quality that packaged. Extracellular polysaccharides layer (e.g. cellulose), synthesized by Acetobacter xylinum and potentially become the material of intelligent packaging, because it was good to absorb pH indicator dyes. Nitrogen source added differently in the medium of Acetobacter xylinum will gives some cellulose different characteristic too. The aim of this research was to determine the effect of Ammonium sulphate and yeast extract addition to cellulose characteristic as the material of intelligent packaging. Bacterial cellulose used to immobilized pH indicator dyes such as Bromothymol Blue (BTB), Phenol Red (PR), Methyl Red (RR), Bromothymol Blue – Methyl Red (BTB+MR), and Bromothymol Blue – Phenol Red (BTB+PR). The intelligent packaging will be applied to fresh meat for 24 hours in room temperature storage. During the storage, fresh meat produces Total Volatile Bases Nitrogen (TVBN), TVBN increase during deterioration and accumulated in the package until pH increases and detected by indicator due to color changes. The result shows that utilization of nitrogen source (Ammonium sulphate) produce better characteristic of bacterial cellulose so that the bacterial cellulose used for intelligent packaging. Furthermore, immobilization solution of BTB on pH 2.74 and PR on pH 2.66 in bacterial cellulose shows significant color changes that can visually observe to quality changes of fresh meat.

1. Introduction
Intelligent packaging (smart packaging) has an external and internal indicator to detect the history of food quality that packaged [1]. FQI (Food Quality Indicator) is one of intelligent packaging that used for monitoring food quality, FQI got color changes as the effect of chemical and biological reaction in the package and indicates product deterioration [2]. Polymer membrane based on bacterial cellulose used as the basic material of intelligent packaging. Bacterial cellulose has unique nano-fibrillar structure so it has porosity, tensile strength, high water binding capacity, good biocompatibility, high purity, hemicellulose, lignin, non-cellulose compound free, biodegradable, and renewable [3,4].
Intelligent packaging based on bacterial cellulose was researched before by using Bromophenol blue pH solution in guava and mangoes [5,6]. Fresh meat is one kind of perishable food, a smart indicator used to identify fresh meat spoilage gradually inside the package. During storage, fresh meat releases some peculiar odor due to volatile amine forming as known as Total Volatile Bases Nitrogen (TVBN), and volatile organic compound (e.g. H₂, CO₂, H₂S, NH₃, CH₄) increases during fresh meat deterioration. Increasing of TVBN caused by microorganisms activity [7]. The Increment of pH due to TVBN that accumulated inside the package, then it detected by the indicator and causes color changes in pH indicator visually [8]. Dyes indicator used as microbial metabolites indicator to monitor fresh meat deterioration or food spoilage [9]. Generally, pH indicator immobilized in polymer cellulose membrane [8]. Various polymeric and biopolymeric materials have been utilized as solid support of pH indicators for smart packaging. However, bacterial cellulose film has a potential to replace synthetic polymers due to its high chemical purity, high mechanical stability, strength and other excellent qualities.

Bacterial cellulose is a natural biopolymer like extracellular polysaccharide that synthesized by various kind of bacteria such as Acetobacter, Rhizobium, Agrobacterium, and Sarcina [10]. Among of the bacteria, Acetobacter xylinum strain is the most effective to produce cellulose and capable of growing in the various substrate such as glucose, sucrose, invert sugar, fructose, ethanol, and glycerol to produce cellulose. Acetobacter xylinum capable of oxidizing glucose becomes a long polymer such as polysaccharide or cellulose, that was white fiber that formed during gradually fermentation process. In primary metabolites system, these bacteria produce acetic acid, water, and reusable energy in its metabolisms. Acetobacter xylinum belongs to Acetobacter genus which is gram-negative, cannot change into endospore form, obligate aerobic, capable of oxidizing ethanol, and produce acetic acid [11]. Acetobacter xylinum is tolerant to acid, grows on pH 3.5 – 7.5, and form cellulose optimally if grown on pH 4.0 and 5.0 at 28 – 30°C.

Hestrin Schrammm medium introduced by Hestrin and Schramm in 1954 as a fermentation medium (synthetic) for Acetobacter, used for forming bacterial cellulose membrane by Acetobacter xylinum. However, it takes a plenty cost on the making of Hestrin Scharmmm medium. Alternatively, coconut water can be used as a natural medium to produce bacterial cellulose membrane. The production of bacterial cellulose that grown in coconut water known as nata de coco that can consume as functional food like good dietary fiber for digestive systems. But, it was different to cellulose that grown in synthetic medium, because it can’t be consumed and the production of cellulose yield tend to be a bit.

Coconut water contains some nutrition such as C, N, and mineral needed for Acetobacter xylinum growth. However, a macro substrate like C and N necessarily added to nata could be produced optimally during fermentation. sucrose, glucose can obtain Carbon sources and flour added. Meanwhile, nitrogen sources can be obtained by urea, Ammonium sulphate (ZA), and yeast extract [12].

The aim of this research was to know the potential and characteristics of bacterial cellulose that produced by coconut water medium with different nitrogen sources as a basic material of intelligent packaging indicator that will be applied on fresh meat. Bacterial cellulose characteristics will be determined by the yield, moisture content, and thickness.

2. Materials and Method

2.1. Materials and tools
Tenderloin 200 g purchased from Tamangapa Raya slaughterhouse, coconut water used as natural medium for Acetobacter xylinum fermentation, food grade Ammonium sulphate (ZA) and yeast extract used as nitrogen sources, Acetic acid 96%, Acetobacter xylinum cultures, NaOH 5%, sucrose, Bromothymol blue, Phenol red, Methyl red, glycerol, Styrofoam, wrap plastic, aluminium foil, hair dryer, Erlenmeyer glass, petri dish, pyrex glass, beaker glass, volume pipettes, moisture analyzer.
DSH-10, pH meter (Horiba Laquatwin compact pH meter P-33), oven, analytical balance, digital microscope, micrometer screw (MDC-25M, Mitutoyo, MFG), and camera (Sony powershot S110).

2.2. Methods

2.2.1. Determination of best nitrogen sources in fermentation medium of bacterial cellulose (Acetobacter xylinum).
The aim of this step was to determine the best nitrogen sources that produce bacterial cellulose membrane (nata pellicle) during fermentation in a natural medium, the procedure are coconut water from optimally ripening state coconut (11 – 12 months old) is allowed for 5 days to decrease the pH. The coconut water is stewed and add some sucrose for 5% (w/v), Acetic acid for 1% (v/v), and nitrogen sources for 0.5% (w/v), nitrogen sources used are food grade Ammonium sulphate (ZA) and yeast extract. The hot coconut water as much as 1,200 ml poured into a container sized 19×16.5×9 cm. The surface of the container is covered by aluminium foil or sterile paper. Coconut water in the container is allowed for 24 hours, and inoculate Acetobacter xylinum starter for 10% (v/v). Then, the starter fermented for 4 – 14 days at 28 – 30°C to be formed nata pellicle or cellulose membrane in the surface of the medium. Every step is done aseptically and sterile to prevent contamination. The best bacterial cellulose determined by yield, moisture content, and thickness.

2.2.2. Purification of bacterial cellulose.
Bacterial cellulose like nata pellicle that formed in the surface of the medium is washed in running water and soaked for 2 days with regular water changes. Furthermore, the cellulose is soaked in alcohol 70% for 1 minute, heated in distilled water at 100°C for 20 minutes and reheated in NaOH solution 5% at 100°C for 60 minutes to remove some bacteria cells and substrate attached in cellulose layer. Then, the cellulose is rinsed in running water and soaked for 24 hours with regular water changes until it reaches pH 7.0 or neutral. The purified cellulose looks transparent.

2.2.3. The making of intelligent packaging indicator label.
The purified cellulose layer is allowed for 24 hours in filter cloth until the moisture content is decreased. The half dry cellulose made into strips in size 1.5 cm×4 cm and pressed in the pyrex glass until it becomes flat. The cellulose is dried in an oven at 60°C for 30 minutes. Furthermore, absorption of BTB indicator solution pH 2.74, BTB pH 5, PR pH 2.66, PR pH 5, MR pH 5.06, MR pH 4.69, BTB + MR pH 4.6, BTB + PR pH is done through the soaking of dry cellulose in indicator solution at room temperature (28±2°C) for 24 hours. Indicator solution pH is set using acetic acid for 20% and NaOH for 5% during solution absorption process of cellulose. Then, cellulose washed with distilled water to eliminate color indicator that not attached to cellulose and dried it using a hair dryer.

2.2.4. Application of smart packaging indicator of fresh meat
Fresh tenderloin in normal pH (5.6-5.7) obtained from Tamangapa Raya Makassar slaughterhouse after the slaughter in an early day, slaughter is done in 1 hour postmortem and laid into a specific food box, then the tenderloin is put inside styrofoam box in size 38×29×30 cm that contains some crystal ice. The sample carried to the laboratory and sterile prepared into some pieces (200 g/pack). The meat slices are packaged in styrofoam (2.05 g/cm³), each indicator label placed in a surface of the package that covered by LDPE film. The sample stored at room temperature (28±2 °C) in normal light exposure.

2.2.5. Observation parameters of bacterial cellulose.
Yield, thickness, and moisture content will be observed. Nata yield calculated by the mass of cellulose per medium volume (ml) per 100%. Thickness measured by micrometer screw (MDC-25M, Mitutoyo, MFG) and has accuracy of 0.001 mm. Each of layer measured by 5 different sides, the whole mean of
measurement results is a final result of bacterial cellulose thickness. Moisture content and dry weight of bacterial cellulose also measured by moisture analyzer (DSH-10) method in weight 5g±0.01.

2.2.6. Measurement of the color indicator using color analysis.
The quantification of dyes indicator done by DSLR camera (Canon 1200D) to take an indicator picture. Picture is taken from ±20 cm distance and the distribution of dyes analyzed by Adobe Illustrator CC 2017. The graphics software Adobe Illustrator CC 2017 is used to measure those red values as color value for all membrane color measurements to obtain color distribution, averages, and so on. Eyedropper Tool on Adobe Illustrator CC 2017 is used for the color value of a selected area in the membrane image of the color indicator.

3. Results and discussion

3.1. Physical properties of bacterial cellulose

Physical properties of bacterial cellulose shown in Table 1 involve yield and thickness using two nitrogen sources (Ammonium sulphate 0.5% and yeast extract 0.5 %) has a significant difference. While, the moisture content of bacterial cellulose does not differed markedly from two treatment. Bacterial cellulose with Ammonium sulphate addition as nitrogen sources has 27.7 % of yield mean, and 5.99 mm thickness which is more than bacterial cellulose means with yeast extract added for 9.67% and 1.91 mm. Based on some research, at ZA for 0.5 % bacterial cellulose thickness is the thickest and tend to decrease with some addition of ZA until 0.75 % while using yeast extract doesn’t give any significant impact to yield and thickness of bacterial cellulose [13,14]. Nitrogen content like Ammonium sulphate as much as 20-21 % will be assimilated with another compound in medium to support some nutrition needs of Acetobacter xylinum [15].

Table 1. Physical properties of bacterial cellulose with nitrogen source added Ammonium sulphate 0.5% and yeast extract 0.5 %.

| Nitrogen Source     | Yield (%)     | Moisture content (%) | Thickness (mm) |
|---------------------|---------------|----------------------|----------------|
| Yeast extract       | 9.67 ±3.06a   | 88.47 ±0.93a         | 1.91±0.99a     |
| Ammonium sulphate   | 27.27 ±1.55b  | 86.04 ±0.77a         | 5.99 ±1.34b    |

Assays were performed in triplicate. Mean ± SD value in the same column with different superscripts are significantly different (p≤ 0.05)

Thickness has positive correlation with the yield, the thicker Ammonium sulphate produced, the higher is the yield. The enhancement of Ammonium sulphate will increase the thickness and yield of bacterial cellulose, because nitrogen is needed by Acetobacter xylinum as cellulose biosynthesis component. However, excessive addition of nitrogen sources will decrease yield and thickness of cellulose. Ammonium sulphate has SO\(_4^2\) ion which is acidic, excessive use of Ammonium sulphate due to drastic pH decreases of medium so fermentation is too acidic and interfere Acetobacter xylinum activity. Based on some research, addition of Ammonium sulphate more than 1 % will decrease production of nata de coco yield [16]. Moisture content of bacterial cellulose with Ammonium sulphate and yeast extract added has no different at all, each of them has value 86.04 % and 88.47 %. Bacterial cellulose with a good quality has more than 85% of moisture content, bacterial cellulose cavity filled of water during pellicle layer forming. The more water trapped in cellulose pellicle structure, the more thicker is bacterial cellulose. Other than that, Figure. 1 shows different looks of extract yeast and ammonium sulphate addition as nitrogen sources of bacterial cellulose. The addition of ammonium sulphate produces clean white bacterial cellulose, just like bacterial cellulose color in general. meanwhile, the addition of yeast extract produces orange color of bacterial cellulose.
Figure 1. Bacterial cellulose formed after 7 days of fermentation with Ammonium Sulphate addition (left) and yeast extract (right).

3.2. *The Response of smart packaging toward fresh meat spoilage*

Figure 2. Application of intelligent packaging indicator based on bacterial cellulose with Ammonium sulphate added as nitrogen sources, at 0 hour (a), at 8 hours (b), at 16 hours (c), and 24 hours (d) with dyes indicator from top left to bottom right such as BTB+PR pH 5, MR pH 4.69, PR pH 2.66, BTB pH 2.74, BTB pH 5, PR pH 5, MR pH 5.06, and BTB + MR pH 4.6.
TVBN (Total Volatile Bases Nitrogen) compound is the result of protein decomposition by bacteria and enzyme activity that produce ammonia and CO$_2$. That compound contains base and volatile nitrogen due to pH enhancement and bad odor of meat. TVBN on fresh meat increase over time during storage. Forming of volatile base will accumulated in package and react to intelligent packaging indicator so that color changes of indicator easy to observe visually. Long storage of fresh meat and indicator color changes shows linear correlation, that the longer time of meat storage, the more obvious color changes of package indicator. Range of indicator color (Δ red) changes in Figure 2 and Figure 3 shows that all of the indicators capable to detect pH changes through color changes. However, not all color changes of indicator can easily visually observed. BTB solution on pH 2.74 and PR solution on pH 2.66 has higher sensitivity than PR on pH 5, BTB on pH 5, MR on pH 4.69, MR on pH 5.06, BTB-PR on pH 5, and BTB-MR on pH 4.6. BTB on pH 2.74 and PR on pH 2.66 gives a good results and the color changes can be visually observed since 8 hour of storage, color changes from yellow to a bit red occurs to PR on pH 2.66, while color changes from yellow to a bit green occurs to BTB on pH 2.74. In 16 hours of storage, color changes from yellow to red occurs to PR on pH 2.66, while color changes from yellow to green occurs to BTB on pH 2.74

4. Conclusion

The results presented in this study show that Ammonium sulphate 0.5% addition as nitrogen sources produce good physical properties of bacterial cellulose than yeast extract addition. Based on T-test result, bacterial cellulose with ammonium sulphate addition has 86.04±0.77 mm of thickness mean and 27.27±1.55% of yield that differs markedly with yeast extract addition treatment. Therefore, bacterial cellulose that obtained from Ammonium sulphate addition used as basic material of intelligent packaging label. Based on observation results of intelligent packaging indicator label based on bacterial cellulose, that bromothymol blue (pH 2.74) and phenol red (pH 2.66) has sensitive color changes...
changes to packaged fresh meat deterioration. Color changes of indicator label seems obvious at 16 hours of storage, bromothymol blue indicator label has color changes from oranges to green while phenol red has color changes from oranges to red.

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