Development of edible films from tapioca starch and agar, enriched with red cabbage (*Brassica oleracea*) as a sausage deterioration bio-indicator

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**Abstract.** Sausage spoilage has been identified as a cause of some food poisoning cases. Development of a bioindicator film is one of the alternative methods to detect sausage deterioration. The objectives of this paper were to develop a bioindicator edible films (BEF) from tapioca starch (TS), agar, and red cabbage juice (RC), and to evaluate its performance on sausage deterioration detection. The experiment had a 3x3 randomized factorial experimental design (agar: 3, 5, 7% by weight of TS; RC: 10, 15, 20% v/v based on 100% of suspension). Glycerol was used as the plasticizer. The results showed that the addition of agar into the film solution increased the thickness, elongation, and tensile strength, and decreased water vapor transmission rate (WVTR). While the addition of RC increased the thickness, but decreased elongation, tensile strength, and WVTR. BEF consisting of 2% tapioca starch, 7% (w/w) agar and 10 % (v/v) RC was chosen to apply on sausage. It could detect an increase in the microbial population and in the pH variations as result of sausage deterioration at 24, 48, and 72 h shown through color changes of BEF from bright purple at 0 h to light purple, dark purple-blue, and purple-green color respectively.

**Keywords:** edible film, sausage, spoilage, red cabbage, bioindicator

1. Introduction

Food poisoning cases caused by food spoilage are an important cause of morbidity and mortality in Indonesia. The updated data from Indonesia National Agency of Drug and Food Control (BPOM) (2017) noted 19 food poisoning incidents in January - March 2017 [1]. One of the most commonly reported source of food poisoning in Indonesia are meat-based foods such as sausage, meatballs, etc. In August 2016, at least 27 students of an elementary school in Sragen, Indonesia got food poisoning from eating contaminated sausages. Either meat or meat products provide excellent growth media for both bacteria, yeasts and molds [2].

Intelligent packaging is a concept that has been developed to detect, to sense, to record, to trace and to monitor the condition of packaged products [3,4]. Edible films have a great chance as an alternative for intelligent and eco-friendly packaging to detect sausage spoilages. Edible-based intelligent packaging based on edible films from tapioca starch, agar, and red cabbage with enhanced functions as sausage spoilage bioindicator have been studied. The main advantage of applying bioindicator films is to easily monitor the level of spoilage of packaged food products in a nondestructive manner during distribution and retail sale [5]. Moreover, biofilm is known as the future of eco-friendly packaging. Tapioca starch has an increasing potential for biofilm production in Indonesia. Asante-Pok, (2013) reported that Indonesia is the second world’s largest producer of cassava after Nigeria [6]. Starch is a natural polymer that has poor physical properties. Incorporation of materials such as agar has been applied as a means to improve the properties of starch. Agar has repeating units of D-galactose and 3,6-anhydro-L-galactose and low content of sulfate esters and has the ability to form strong gels.
characterized by melting points far above the initial gelation temperature [7,8]. Previous research revealed that agar based films could improve starch properties such as increasing both tensile strength and elongation, water permeability, decreasing the film solubility, and resistance to heat [8]. Moreover, the addition of anthocyanins from red cabbage into the polymer materials to create bioindicator edible films (BEF) offers an extra advantage. Anthocyanins changes a color due to its phenolic or conjugated substances based on variation in pH.

Color based pH indicators are used to indicate microbial metabolites because a microbiological growth could induce a pH change [9]. Metabolism of amino acids by some spoilage bacteria used as a meat spoilage indicator are NH₃, amines, and other basic compounds resulting in a pH increase close to 7.0-8.0 [10]. S. aureus, E. coli and Pseudomonas spp. are some spoilage microorganisms responsible for meat spoilage. The objectives of this paper were to develop BEF from tapioca starch, agar and red cabbage juice, and to evaluate its performance on sausage deterioration detection.

2. Experimental
2.1. Materials
Tapioca starch (commercial grade, Rose brand), red cabbages (RC), and fresh beef meat were purchased from a local wholesale market in Malang, Indonesia. Commercial agar powder (particle size 80 mesh, moisture 20%, and yellowish color) was purchased from Golden Agar Sentosa (Surabaya, Indonesia). Glycerol used as the plasticizer was purchased from Merck (Germany).

2.2. Preparation of beef sausages
Sausage making was started with the preparation of the material. The meat was crushed by using a food processor and 5% tapioca starch, 10% egg whites, 10% chopped ice cubes, 2% garlic, 0.5% white pepper, 0.5% coriander, and 1% salt were added, with the percentage of ingredients based on 100% of meat. The mixed dough was put into a stuffer and then filled into casings. Later, the pieces of sausages were boiled for 30 minutes at a temperature of 70-80°C and were cooled to ambient temperature before they were wrapped with BEF for testing.

2.3. Preparation and formation of bioindicator edible films (BEF)
Film solutions were prepared by adding 2% TS, glycerol (30% by weight of TS), agar with different ratios (3%, 5%, 7% by weight of TS) in distilled water and blending with constant magnetic stirring at room temperature for 20 minutes until complete dissolution. The mixture was heated and gelatinized to 90°C with continuous stirring for 10 min before it was cooled to room temperature. Then, the anthocyanin agents from RC (10%, 15%, 20% v/v based on 100% of suspension) were added to film solutions and blended with constant stirring for 10 minutes. The anthocyanin agents were obtained by crushing red cabbage-distilled water (1:2) and filtering on Whatman paper. The final solutions (30 ml) were poured into casting plates (7 cm x 15 cm) and placed in an air-circulating oven at 50°C until they were dry (about 14 - 15 h). The cast films were cooled to ambient temperature before peeling the films off the plates and kept in a plastic bag. The different formulas of the BEF were as in the following table.

| Sample | TS (%) | Agar (% w/w) | RC (% v/v) |
|--------|--------|--------------|------------|
| 1      | 2      | 3            | 10         |
| 2      | 2      | 3            | 15         |
| 3      | 2      | 3            | 20         |
| 4      | 2      | 5            | 10         |
| 5      | 2      | 5            | 15         |
| 6      | 2      | 5            | 20         |
| 7      | 2      | 7            | 10         |
| 8      | 2      | 7            | 15         |
| 9      | 2      | 7            | 20         |
2.4. **BEF characteristics**

Film thickness was determined using a Micrometer Digital Kincrome with an accuracy of 0.01 ± 0.004 mm at five random positions of the film sample. This measurement was performed in three replicates of each sample.

Tensile strength and percent elongation of the films were analyzed using Microcomputer Controlled Universal Testing Machine (model WDW 5E of Time Group Inc, China) following the ASTM D 638 - 99 method (1999) with initial grip at 17 mm and tensile speed of 20 mm/min [11]. The film was cut into 2cm x 7cm strips. Three replicates of each sample were measured. Tensile strength value was calculated by dividing the maximum load by the specimen area of the film using the following equation:

\[
\text{Tensile strength} = \frac{F}{A}
\]

Where \( F \) is the force maximum at rupture of the film in N; \( A \) is initial cross-section area of the film in \( \text{mm}^2 \).

While, the percent elongation was calculated from the maximum elongation during testing by the following equation:

\[
\% \text{ Elongation} = \frac{L - L_o}{L_o} \times 100
\]

Where \( L_o \) is initial gage length; \( L \) is the final length.

The water vapour transmission rate (WVTR) was measured following ASTM D1249-90 method (1993) [12]. Cylindrical films with diameter of 30 mm were placed on the surface of the cups filled with silica gel and coated with paraffin wax around the cups circumference. The WVTR of each film was recorded in glass desiccators containing distilled water to provide 75% RH and measured at 25 ± 2°C. The cups were weighed at different time intervals. This measurement was conducted in three replicates of each sample. WVTR was calculated by dividing the difference of initial and end weight by the time of transfer multiplied by the film area exposed to water surface:

\[
\text{WVTR} = \frac{dw}{A \cdot dt}
\]

Where \( dw \) is difference of initial and end weight in g; \( A \) is film area exposed to water in \( \text{m}^2 \); \( dt \) is difference of initial and end time for 24 h.

2.5. **BEF performance on sausage deterioration**

The BEF was cut into square shaped 6-7 cm\(^2\) in area. Then, the sausages (4-5 cm in length) were coated with BEF and were kept in a room with a controlled temperature of 28°C throughout the test period (0-72 h). The evaluation of BEF color changes was measured each day using a Chroma Meter Minolta CR-300 based on the CIE color system, where color coordinates range from \( L^* = 0 \) (black) to \( L^* = 100 \) (white), \(-a^* \) (greenness) to \(+a^* \) (redness), and \(-b^* \) (blueness) to \(+b^* \) (yellowness). The values of the rectangular coordinates \((L^*, a^*, b^*)\) were used to calculate the total color difference \((\Delta E)\) using following equation [13]:

\[
\Delta E = (\Delta L^*^2 + \Delta a^*^2 + \Delta b^*^2)^{1/2}
\]

Where \( \Delta L^* \), \( \Delta a^* \) and \( \Delta b^* \) are the color parameter values of the difference between color values of the sample at different time intervals (24, 48, 72 h) and color values of the sample (0 h).

Moreover, pH and microbial count determinations of beef sausages were performed each day. The pH was measured using a “Hanna” pH meter calibrated at pH 4 and 7 prior to measurement. A penetration electrode was inserted on the surface of each block of sausages and pH value was read. The microbial count determination was performed based on Bacteriological Analytic Methods (1992) [14]. In brief, 25 g of sausages were weighed and homogenized in 225 ml 0.1% peptone water. The mixture was pipetted into 9 mL of 0.1% peptone water to obtain 10\(^{-1}\) dilution. The solution was further step-wise diluted up to 10\(^{-7}\) dilution. The total plate count was determined using the pour plate method and plate count agar as the medium. Incubation occurred at 37°C for 48 h. Microbial counts were reported as log CFU/g (colony forming units per g of sample).
2.6. Statistical analysis
The experiment had a 3x3 randomized factorial design (agar: 3, 5, 7% by weight of TS and RC: 10%, 15%, 20% v/v based on 100% of suspension). The statistical analyses of the results were performed by analysis of variance (ANOVA) using SPSS (Statistical Product and Service Solutions) version 16.0 and the difference between treatment effects was tested using Duncan’s Multiple Range Test (DMRT). Significant difference was considered at 95% probability. Moreover, the determination of the best BEF was performed using Multiple Criteria Decision Making (MCDM) before wrapping on sausages [15].

3. Result and Discussion
3.1. BEF characteristics
The thickness of BEF was found to be between 33.89 to 38.78 μm as tabulated in Table 2. The thickness of edible films increased significantly (p < 0.05) by incorporating both agar at all concentration and RC 20% v/v, but there were no interactions between both factors. Films thickness can be related to the base material effect features and the interaction between components [16]. The results are similar with the studies of the addition of glycerol in agar-based edible films and sago starch-alginate films incorporated with calcium chloride [8,17]. These results indicated that the agar and RC component in the BEF was solubilized into the solution, thus increasing the thickness of the film.

The WVTR value should be as low as possible for optimal results, because the main function of a food packaging is to avoid or at least to decrease moisture transfer between the food and the surrounding atmosphere, and to decrease the role of water in deteriorative reactions in foods [18,19]. The result of WVTR of the different BEF ranged from 35.42 to 41.84 g.m⁻².24h⁻¹ as presented in Table 2. Japanese Industrial Standard required the maximum WVTR value of a good film to be 10 g.m⁻².24h⁻¹. Therefore, the WVTR values of the current study are not able to meet a good film characteristic [20]. Incorporation of RC into BEF formulation at all levels (10, 15, 20%) reduced WVTR value but not significantly. On the other hand, the addition of agar at all levels (3, 5, 7%) reduced WVTR significantly (p < 0.05). There were no interactions between factors. It is consistent with Abdou and Sorour (2014) that the combination of carrageenan molecules, tapioca starch and glycerol, which binds both to the BEF, caused low WVTR [21]. The WVTR depends on many factors, such as the integrity of the film, the ratio between crystalline and amorphous zones, the hydrophilic/hydrophobic ratio, and the polymeric chain mobility [22].

| Sample | Thickness (μm) | Tensile strength (Mpa) | Elongation (%) | WVTR (g.m⁻².24h⁻¹) |
|--------|----------------|------------------------|----------------|----------------------|
| 1      | 33.89 ± 1.26   | 2.21 ± 0.15            | 7.57 ± 0.29    | 41.84 ± 2.11         |
| 2      | 34.33 ± 1.53   | 2.07 ± 0.21            | 7.41 ± 0.38    | 41.31 ± 2.15         |
| 3      | 35.00 ± 1.20   | 1.80 ± 0.15            | 7.1 ± 1.09     | 40.87 ± 1.84         |
| 4      | 35.44 ± 1.07   | 3.43 ± 0.25            | 8.83 ± 0.11    | 39.36 ± 0.79         |
| 5      | 36.11 ± 0.77   | 3.05 ± 0.17            | 8.63 ± 0.08    | 38.57 ± 2.14         |
| 6      | 36.44 ± 0.77   | 2.82 ± 0.25            | 8.48 ± 0.11    | 37.59 ± 0.85         |
| 7      | 37.22 ± 0.51   | 4.07 ± 0.17            | 10.08 ± 0.57   | 36.62 ± 0.74         |
| 8      | 37.56 ± 0.19   | 4.04 ± 0.25            | 10.04 ± 0.57   | 36.00 ± 0.98         |
| 9      | 38.78 ± 0.19   | 3.63 ± 0.25            | 9.83 ± 0.58    | 35.42 ± 0.93         |

Means followed by different letters (small: among different RC concentration; capital: among different agar concentration) indicate statistically significant differences (p < 0.05).

The mechanical properties of eco-friendly films are of great importance due to their influence on product performance and their potency to protect the integrity of foods. Tensile strength is defined as the maximum tensile stress that the films sustain when forces are being exerted on the film continuously [23]. While elongation is defined as the parameter to determine the film’s flexibility and elongation capacity before the films break [24]. Table 2 shows the mechanical properties of the different BEF.
Tensile strength and elongation are ranging from 1.80 to 4.07 Mpa and 7.1 to 10.08%, respectively. Those mechanical properties are not able to reach Japanese Industrial Standard for elongation that state the minimum of tensile strength and elongation are 3.92 Mpa and 70% respectively [20]. Statistically, the addition of RC at only 20% significantly decreased tensile strength of the films. It was presumably due to interactions between polymer chains and RC juice which interfere with intermolecular bonds, providing discontinuities in the matrix. While, incorporation of agar both 3, 5, or 7% increased both tensile strength and elongation values significantly (p < 0.05). There were no interactions between such factors on both tensile strength and elongation. In general, the addition of agar provides strong bonds and higher tensile strength. The same result was found by several researchers that increasing the concentration of agar in the manufacture of agar-based and potato starch-based films, respectively caused tensile strength increases due to the formation of hydrogen bonds between starch and agar molecules, and yielded compact structures of the films [8,25]. Moreover, water holding capacity of the films might be increased with agar concentration in the film solution thereby enhancing elongation.

3.2. BEF performance on sausage deterioration
The BEF which had the best characteristics was selected before wrapping on sausages. The best BEF was obtained by using the method according to MCDM [15]. The best treatment was obtained in sample 7 (2% TS + 7% agar w/w + 10% RC v/v), as can be seen from the value of each parameter in Table 2. Determination of the best treatment was aimed to choose a sample which has the best properties before wrapping on sausages.

The total color difference (ΔE) is a useful parameter to evaluate the ability of the human eye to distinguish color differences without using a sensory analysis panel [9]. It is considered to be visually perceptible when ΔE > 1 and clinically acceptable when ΔE > 3.3 [26,27]. ΔE showed significant changes during storage of sausage at 24 h and 48 h compared to 0 h, considered as the control. The magnitude of ΔE values found in this research was served as intelligent packaging. It indicates that consumers should be able to distinguish changes in film color of the product packaging during storage. Color differences of BEF #7 are shown in Table 3 and Figure 1. The changes in ΔE may be due to the changes in the anthocyanin structure contained in RC juice. Several factors influence the anthocyanin color, including its own chemical structure (number of hydroxyl groups, methylation degree and glycosylation), pH, temperature, light, presence of oxygen, enzymatic degradation; and interactions with other substances such as sugars, metal ions, and co-pigments [28,29]. At acidic pH environments (1.0-3.0), anthocyanins display purple and red colors, which is mainly in the form of the flavlyium cation and the colorless carbinol base. At pH 2.0-4.0, anthocyanins exist mainly in the quinoidal blue species. Increasing the pH to 5.0 and 6.0 provides a decrease in the color intensity and the concentration of the flavlyium cation, which undergoes hydration resulting a colorless carbinol pseudobase and chalcone. A purple quinoidal anhydrobase and a deep blue ionized anhydrobase are formed at pH > 7.0 and pH > 8.0 respectively, when the equilibrium is shifted. A yellow chalcone is generated through opening of the central pyran ring of carbinol at even higher pH [30,31,32].

Figure 1. BEF #7 color response in contact with representative sausages during storage
Results of ΔE (Table 3) were consistent with the films’ visual appearance (Figure 1). Considering Figure 1, representative visual color changes of BEF during sausage storage showed the obviously detectable. This bioindicator displayed a color change easily visible to the naked eye. When BEF was in contact with sausage at 0 h, it showed a bright purple color, while at 24 h it turned into a light purple color. At 48 h it was changed to a dark purple-blue color and at 72 h it appeared a purple-green color.

The pH is widely used to monitor the shelf life and deterioration of meat such as chilled pork and chicken nugget [33,9,34]. With complete deterioration, the meat pH can reach close to 7.0-8.0 [10]. As shown in Table 3, the pH of the sausages gradually and significantly increased at the last storage time. The pH value of sausage was strongly influenced by the long treatment of storage time. Similar patterns were reported by Mano et al. (2002) and Golasz et al. (2013) for chilled pork meat [35,9]. During storage, meat proteins are degraded by endogenous and microbial enzymes resulting in ammonia and amines, increasing pH [36,10]. This pH increase coincides with microbial counts (see below), and is consistent with the ΔE and visual color changes observed.

| Time (h) | ΔE        | pH         | Microbial counts (log CFU/g) |
|---------|-----------|------------|-----------------------------|
| 0       | 0         | 5.8 ± 0.1  | 4.49 ± 0.02                 |
| 24      | 13.66 ± 0.43 | 5.85 ± 0.13 | 7.81 ± 0.02                 |
| 48      | 17.42 ± 1.13 | 6.12 ± 0.25 | 8.99 ± 0.01                 |
| 72      | 22.47 ± 1.88 | 7.12 ± 0.21 | 9.46 ± 0.03                 |

Means followed by different letters in the same column indicate statistically significant differences (p < 0.05).

During the sausage storage, the microbial counts increased by approximately 5 logarithmic cycles to more than 10^6 CFU/g, as shown in Table 3. Meat products such as sausage are a nutritious, protein-rich food able to support the growth of many types of microorganisms causing it to be highly perishable and having a short shelf-life [37,38]. There was a significant increase in total number of colonies for all storage times. Meat spoilage is a phenomenon resulting in the formation of odors, slime, and gas production. Substances responsible for undesirable odor in spoiled meat are mono-, di-, and trimethylamines, ethyl- and propyl-compounds, ammonia, H₂S, alcohols, and short chain fatty acids [40,2,39]. The most frequent bacteria to occur in and on meat are groups of bacteria Pseudomonas spp., Enterobacteriaceae, Brochothrix thermosphacta, and lactic acid bacteria [41]. It was beyond the scope of the study to characterize the microbial counts in detail. Indonesia National Agency of Drug and Food Control required that a sausage product could not be consumed if it presents a count higher than 5 log CFU/g.

4. Conclusion

Different BEF were prepared by casting a blending solution of 2% tapioca starch, with different amounts of agar and red cabbage juice, as anthocyanin agent. The best improvement in film characteristics based on MCDM method was achieved at 2% TS + 7% agar w/w + 10% RC v/v, which has a thickness of 37.22 μm, tensile strength of 4.07 MPa, elongation of 10.08%, and WVTR of 36.62 g/m²·24h. This best film was successfully able to detect sausage deterioration during storage through changes in the film color that could be detected by the human eye. The results suggest that BEF may be used as breakthrough intelligent packaging for perishable foods in a nondestructive manner. However, further studies are needed to improve physical and mechanical properties of films and to study the anthocyanin stability during storage.
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