Physico-chemical and antimicrobial properties of casein-chitosan edible films as food quality and food safety

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Abstract. Edible films can be useful in extending product shelf life and the surrounding environment to enhance the quality of food products protecting from physical, chemical, biological deterioration. Casein and chitosan are natural materials for producing edible films. These films are also able to incorporate as barriers to water vapor, oxygen and carbon dioxide and as carrier substances to inhibit pathogenic and spoilage microorganisms. Observations were conducted to the formulations of the casein and chitosan solution ratio 1:1; 1:2; 1:3 and 1:4 (ml:ml) in order to determine one that yields the best solution. The objectives of this study was to characterize physicochemical (water vapor permeability, water activity, and film solubility) and antimicrobial properties of casein-chitosan (Staphylococcus aureus, Lactobacillus bulgaricus, Escherichia coli, and Salmonella sp.). The effect of the casein-chitosan on edible films was monitored. The best solution on casein:chitosan ratio = 1:4 (ml:ml). The greatest water vapor permeability with 0.1140 g.mm/m².h.kPa, film solubility with 32.809% and water activity with 0.709. Antimicrobial activity of edible films casein-chitosan against Staphylococcus aureus, E.coli, Salmonella sp. and Lactobacillus bulgaricus was ratio solution dependent.

1. Introduction

Food packaging is urgently needed to prolong the life of food products, protect food products from microorganism contamination and maintain the quality of food products. One of the materials that have the potential to be used for the manufacture of edible films is casein. Casein is a member of hydrocolloid group. Among its many properties is that it is heat stable which makes casein a good ingredient in the making of the edible film [1], this protein has special properties because it is difficult to split by high heat. Unfortunately, casein does not have the ability to form a gel, so the resulting film is not elastic. Adding chitosan will result in gel formation. Chitosan is a type of chitin-derivative polysaccharide Chitosan coating is strong, elastic, and flexible. Further, it is also edible which makes this coating environmentally friendly packaging material [2].

The component of plasticizer is needed to help increase binding between protein molecules in the film, but the amount cannot be too little or too much [3]. If too little the produced films are fragile and if too much, the value of unwanted WVP films will be high resulting in films that are too elastic. Chitosan has good ability in binding water and oil because of its high chemical reactivity property which is supported by the presence of polar and nonpolar groups. This is the reason why chitosan can be used as an excellent thickening or excellent gelling agent, as a binder, stabilizer and texture builder [4]. This shows that chitosan can reduce water activity (Aw). Research by [5], natural galactomannans are an
alternative material that can be for the production of edible films/coatings based on their edibility and biodegradability.

The physical properties of edible films include tensile strength, elasticity, thickness, water vapor permeability, solubility, transparency, and surface appearance of the film [6]. It is necessary to add other ingredients such as chitosan with the right ratio in making edible films that have antimicrobial activities [7]. The antimicrobial activity of chitosan has been observed against a wide variety of microorganisms including fungi, algae, and bacteria [8] and [9].

Chitosan has many amine groups along its chain (cationic). The group -NH$_2$ on chitosan when reacted with acid changes to –NH$_3^+$. The positive charge of the NH$_3^+$ group on chitosan is able to interact with the negative charge on the surface of the bacterial cell, which is made of theicoic acid on Gram positive bacteria and lipopolysaccharide in Gram negative bacteria. This interaction is expected to interfere with the formation of peptidoglycan so that the cell has no sturdy sheath thus is subject to lysis. This means the metabolic activity will be hampered and eventually end up in death [10]. Additionally, when compared to other bio-based food packaging materials, chitosan has the advantage of antibacterial activity [11 and 12]. Chitosan edible films can protect food, prevent contamination of microorganism contamination, and reduce gas and aroma transfer, as well as able to block the transfer of oil, oxygen, and water vapor [2 and 13], suggested that chitosan inhibits the growth of Gram-negative and Gram-positive bacteria. The antimicrobial mechanism works by damaging the structure of cell membranes and walls of microorganisms. This study was aimed at determining the best ratio between casein and chitosan in the manufacture of edible film. The ratio is expected to produce an effect in terms of physicochemical quality and antimicrobial activity in the edible film formed. The best ratio between casein and chitosan can be used as a reference in producing edible film and coating.

2. Materials and method

The materials used in the manufacture of edible films were casein (Merck), chitosan (Makmur Sejati), glycerol (Merck), beeswax (Rimba Raya), aquadest, and 2% acetic acid. Solubility test: aquadest and filter paper. WVP test: aquadest. Aw: solution of BaCl$_2$ and 2H$_2$O. Antimicrobial activity test: Staphylococcus aureus culture, Escherichia coli culture, Salmonella sp. culture and Lactobacillus bulgaricus culture, media Baird-parker Agar Base (OXOID), Eosin Methylene Blue Agar (Modified) Levine (OXOID), Salmonella shigella Agar (OXOID), MRS Agar (De Man, Rogosa, Sharpe) (OXOID), and aquadest.

2.1. The manufacturing process

First, 2.5 g casein was dissolved in 100 ml aquadest for about 30 minutes at ≤ 50°C and then was added glycerol 0.28% for solution during the heating process. Second, 2% acetic acid was added to chitosan, then the chitosan was dissolved in 98 ml aquadest for 30 minutes at ≤ 50°C and then was added glycerol 0.28% for solution during the heating process. The next step was the mixing of casein and chitosan solution in different ratios for 60 minutes at 60 °C. After that, 0.5% beeswax was added the solution. The solution was hand-mixed for 10 minutes then measured 25 ml and casted on a petri dish. Edible film solution was casted in a petridish and dried at room temperature for 72 hours. The ratio treatments given in this study were four treatments with five replications, as follows:

CS1 = Casein Solution 1 : 1 Chitosan solution
CS2 = Casein Solution 1 : 2 Chitosan solution
CS3 = Casein Solution 1 : 3 Chitosan solution
CS4 = Casein Solution 1 : 4 Chitosan solution

2.2. Solubility test

Measurement solubility of the edible film [14], samples were cut to a size of 3x2 cm$^2$. Samples with filter paper are dried at 105°C, for 24 hours. Filter paper and samples are weighed separately, to determine the initial weight of the sample (W1). The sample was put into a 50 ml centrifuge tube containing 10 mL of distilled water. Soaking is carried out for 24 hours at room temperature and stirred
slowly periodically using a shaker. The solution is filtered, then filter paper and insoluble film are dried using an oven at 105°C for 24 hours. The sample is weighed (W2) to determine dry material that is not soluble in water. Solubility is calculated using the formula:

\[ \text{Solubility (\%)} = \frac{(W1 - W2)}{W1} \times 100\% \]  

(1)

2.3. WVP test
WVP [15] was determined using a cup method at 25°C and 100%/50% RH gradient, following ASTM E 96 (ASTM 2000). Distilled water was placed in each test cup with a 57 mm inside and a 15 mm inner depth. The distance between water and the film was 10.7 mm, and the effective film area was 25.5 cm². Test cup assemblies were placed in the environmental chamber (25°C and 50% RH). Each cup assembly was weighed every hour for 6 h using the electronic balance (0.0001 g accuracy) to record moisture loss over time. Water vapor permeability was then corrected for the resistance of the stagnant air gap between the film and the surface of the water using the WVP correction method. WVP was calculated as follows (modified by Tanaka et al., 2001):

\[ \text{WVP} = \frac{\text{Weight of edible film (g)} \times \text{thickness of edible film (mm)}}{\text{Exposed area of edible film (m²)} \times \text{time of gain (h)} \times \Delta P (KPa)} \]  

(2)

2.4. Water activity
Measurement of water activity [16] using the Aw meter (Water Activity hygropalm tipe HP23 rotronic). The instrument is calibrated by inserting BaCl₂ 2 H₂O fluid and closing it for 3 minutes until the number on the reading scale becomes 0.9. Samples weighed 1 gram. Aw meter is opened and the sample is inserted and the appliance is closed awaited up to 3 minutes. After 3 minutes, the Aw scale is read and recorded, noting the temperature scale and the correction factor. If the temperature scale is above 20°C, then the Aw scale reading is added as much as the excess temperature multiplied by the correction factor of 0.002, as well as the temperature below 20°C.

2.5. Antimicrobial activity test
The tested organisms E. coli and S. aureus were grown in lysogeny broth (LB) and tryptic soy broth (TSB) mediums respectively for 24h at 37°C. The antimicrobial test was carried out according to the method developed by [17] with some modifications. The inhibitory zone test on solid medium was used for the determination of the antimicrobial effects of the films. Edible films were cut into discs (diameter 10 mm), and 2 discs were placed carefully into each petri dish containing solid medium, where 0.1 ml seeding culture had been spread. The concentrations of the E. coli, S. aureus, Salmonella, and L. bulgaricus seeding culture were 2x10⁸ CFU/ml and 2x10⁶ CFU/ml, respectively. The petri dishes were then incubated at 37°C for 24 h in the appropriate incubation chamber. The plates were examined to find the inhibition zone of the film discs. The diameter of the zone was measured with a sliding caliper (Shanghai, China) and the area of the whole zone was calculated.

3. Results and discussion
Data, variance analysis and Duncan's Multiple Distance test (UJBD) on the testing of WVP, solubility and Aw edible films are shown in table 1.
Table 1. WVP, solubility and Aw with a various ratios of casein and chitosan.

| Film Type | WVP (g.mm/m². h. kPa) | Solubility (%) | Aw   |
|-----------|------------------------|----------------|------|
| CS 1      | 0.0168±0.039<sup>a</sup> | 42.034±3.434<sup>a</sup> | 0.732±0.022 |
| CS 2      | 0.0914±0.040<sup>a</sup> | 37.655±1.671<sup>a</sup> | 0.717±0.009 |
| CS 3      | 0.1370±0.037<sup>ab</sup> | 32.641±4.489<sup>ab</sup> | 0.712±0.006 |
| CS 4      | 0.1140±0.057<sup>b</sup> | 32.809±4.131<sup>b</sup> | 0.709±0.006 |

Note: <sup>a,b</sup> Superscript which different at the same column showed very significant differences (<p<0.01) on solubility, and showed significant differences (<p<0.05) in WVP.

3.1. WVP

The result of variance analysis shows that the four ratios were significantly different in WVP test. The difference is caused by ratio of casein and chitosan which was more than 1:1, because chitosan produces a layer above the formed edible casein film. The bigger the chitosan ratio, the more layers that it will produce above the edible casein film. Research by [18] showed that the addition of chitosan fillers at concentrations of more than 75% will result in a greater WVTR rate. The research value of WVP of the edible film with a ratio of casein and chitosan ranged from 0.01-0.114 g.mm/m².h.kPa. This result was in support of several edible film studies with different ingredients. The results of the study by [19] using whey base ingredients with various concentrations of glycerol had an average water vapor transmission rate of 0.0102-0.0149 g/mm²/hour. The highest value of water vapor transmission rate of 8.05 g/mm²/hour was found in the sorbitol plasticizer treatment without the addition of palmitic acid [20]. WVP values increase as chitosan comparisons increase. The results of [18] study showed that glycerol (plasticizer) and chitosan in the manufacture of edible are hydrophilic in nature, so chitosan and glycerol will produce vapor absorption. Film thickness will also affect the mechanical properties of films such as the rate of water vapor transmission. The high value of the water vapor transmission rate is related to the high protein content in the film and film thickness. Films that one thick and with a high amount of protein can absorb more water from the environment [21]. The adding lipid in a composite edible film of whey protein and konjac glucomannan flour may control the transport of moisture in the edible film [22].

3.2. Solubility

The variance analysis results on the manufacture of edible film with various ratio of casein and chitosan showed very significant differences (<p<0.01) in the value of solubility. The stronger the bond of chitine amine group, the lower the solubility. A previous study stated that chitosan is polycationic in acidic media (pKa 6.5) and can interact with negatively charged compounds [23]. The results of the study by [24] showed that the solubility of edible films ranged from 43-56%. The solubility of edible film produced in this study is quite high, ranging from 32-42%. The value of solubility expected from the edible film of this study was that low solubility indicates that edible film was not easily degraded and can be used as primary packaging so that the coating on food is not easily damaged. According to [25] and [26], high solubility causes the edible film to dissolve easily in water and its ability to hold water is reduced.

3.3. Water activity

Variance analysis showed that the four ratios were not significantly different (<p>0.05) in Aw value. The increase of Aw value was not always followed by a decrease in WVP because the water content in food can be either physically or chemically bound, or in the form of free water. Similar to solubility, low Aw value is ideal. This means that microbes cannot grow easily and, thus, the edible film has a long shelf life. The result of Aw test in this study ranged from 0.70 to 0.73. These are minimum Aw values that permit microbial growth, since, for example, bacteria have Aw 0.90; yeast 0.80-0.90; and molds 0.60-
0.70 [27]. In the study, ratio 1:4 produced the lowest Aw value. Higher chitosan content results in low Aw values because water is weakly bound.

3.4. Antimicrobial activity of edible film

Data, variance analysis and Duncan's Multiple Distance test (UJBD) on antimicrobial activity of casein-chitosan edible film against *Staphylococcus aureus*, *Lactobacillus bulgaricus*, *Escherichia coli*, and *Salmonella* sp. can be seen in table 2.

3.4.1. *Staphylococcus aureus* and *L. bulgaricus*. Variance analysis results showed that the four casein-chitosan ratios produced very significant differences (p<0.01) in the inhibition zone diameter against *S. aureus* growth. This means that *S. aureus* were not able to grow around the edible film. Chitosan successfully inhibits the growth of *S. aureus* by forming a polymer membrane on the surface of the cell which prevents nutrients from entering the cell, eventually causing cell lysis [28].

| Edible Film Type | *Staphylococcus aureus* | *Lactobacillus bulgaricus* | *E. coli* | *Salmonella* sp. |
|------------------|------------------------|---------------------------|-----------|-----------------|
| CS 1             | 12.10 ± 0.86<sup>a</sup> | 15.52 ± 1.03<sup>a</sup>  | 21.98 ± 0.92<sup>a</sup> | 16.41 ± 2.66   |
| CS 2             | 13.53 ± 0.99<sup>ab</sup> | 16.92 ± 1.29<sup>ab</sup> | 24.99 ± 3.02<sup>ab</sup> | 18.38 ± 3.00   |
| CS 3             | 16.02 ± 1.07<sup>bc</sup> | 17.74 ± 3.04<sup>ab</sup> | 25.82 ± 2.25<sup>b</sup>  | 21.17 ± 4.64   |
| CS 4             | 18.06 ± 2.45<sup>c</sup> | 19.80 ± 2.67<sup>b</sup>  | 26.92 ± 3.14<sup>b</sup>  | 20.62 ± 5.75   |

Note: <sup>a, b, c</sup> Different superscripts in the same column showed very significant differences (p<0.01) in *Staphylococcus aureus*, and showed significant differences (p <0.05) in *E. coli* and *Lactobacillus bulgaricus*.

Duncan's Multiple Distance test (table 2) shows that there is a very significant difference in the inhibition zone diameter of the edible film against *Staphylococcus aureus* in treatments CS1, CS2, CS3 and CS4. The diameter of the broadest inhibition zone was in CS4 with 18.06 mm, while the narrowest one was found in CS1 with 12.10 mm. Chitosan can also prevent nutrients from entering the cell, thus inhibiting cell reproduction. Hence, the higher the chitosan ratio, the higher the antimicrobial activity against the bacterium. Differences in the diameter of the edible film inhibition zone in *Staphylococcus aureus* growth more clearly can be seen in figure 1.

![Figure 1](image1.jpg)

**Figure 1.** The edible film inhibition zone in *Staphylococcus aureus*.

Variance analysis results showed that the four casein-chitosan ratios produced a significant difference (p<0.05) in the inhibition zone diameter against *L. bulgaricus* growth and successfully inhibits the growth of *L. bulgaricus*. Duncan's Multiple Distance test also shows that there is a significant difference in the inhibition zone diameter of edible film against the *L. bulgaricus*. The broadest inhibition zone diameter was found in CS4 with 19.80 mm, while the narrowest inhibition zone was in CS1 with 15.52 mm. Differences in the diameter of the edible film inhibition zone against *L. bulgaricus* growth can be seen in figure 2.
**Figure 2.** The edible film inhibition zone in *Lactobacillus bulgaricus.*

*Staphylococcus aureus* and *L. bulgaricus* are Gram-positive bacteria, while *E. coli* and *Salmonella* sp. are Gram-negative bacteria. The cell wall of *Staphylococcus aureus* and *L. bulgaricus* is composed of 30-40 peptidoglycan layers [13]. Positive charge from chitosan can be bound to and cause distortion and breakdown of bacteria’s cell walls due to osmotic differences and cytoplasm exudation. Mechanism of the antibacterial action of chitosan is through cross-linkage between the polycation properties of chitosan and the nature of anions on the surface of Gram-positive bacteria which damage cell wall [29]. Cell wall damage prevents nutrients from entering the cell, thus interfering with DNA and RNA synthesis which cause bacterial death [30]. Different result has been obtained where 15% w/w chitosan showed no significant improvement of antimicrobial activity in sweet potato starch film [31]. The four inhibition zone categories are ≤ 5 mm as weak, 5-10 mm as medium, inhibition zone 10-20 mm as strong and ≥ 20 mm as very strong [32]. Based on these categories, the diameter of the inhibition zone for *Staphylococcus aureus* growth as well as for *L. bulgaricus* growth can be categorized as strong.

3.4.2. *Escherichia coli* and *Salmonella* sp. Variance analysis results showed that the four casein-chitosan ratios produced a significant difference (*p*<0.05) in the inhibition zone diameter of *E. coli* growth. Chitosan increases the antimicrobial activity of the edible film that has formed by inhibiting the growth of *E. coli* so that the bacteria cannot grow around the edible film. Chitosan has many amine groups along its chain (cationic) [10]. Group -NH2 in chitosan, when reacted with acid, will change to –NH3+. This interaction will interfere with the formation of peptidoglycan, damaging the integrity of cell membrane and causing exudation of cell organelle. This result in a weak sheath, thus leading to lysis and eventually death. Duncan’s Multiple Distance test (table 2) shows that there are significant differences in the inhibition zone diameter of the edible film against *E. coli* in treatments CS1, CS2, CS3 and CS4. This can be seen from the different notations in each treatment. The broadest inhibition zone diameter was found in CS4 with 26.92 mm, while the narrowest diameter was found in CS1 with 21.98 mm. The inhibition zone diameter of edible film against *E. coli* growth can be seen in figure 3.

**Figure 3.** The edible film inhibition zone in *E. coli.*
Variance analysis on the four casein-chitosan ratios indicated a non-significant difference (P > 0.05) in the inhibition zone diameter against *Salmonella* sp. growth. The result showed that the inhibition zone diameter increased from CS1 to CS3, but it decreased in CS4. This may mean that the inhibition ability of chitosan against *Salmonella* sp. reaches its maximum in CS3. It seems that after reaching a certain concentration, chitosan experiences a decrease in its antimicrobial activity [13]. Chitosan has an ammonium group (nitrogen atom) which functions as a food source for bacteria. Therefore, a concentration that is higher than that of maximum antimicrobial effect will result in excess chitosan which, ironically, feed the bacteria, thus leading to a decrease in antimicrobial activity.

The average inhibition zone diameter of the casein-chitosan edible film against *Salmonella* sp. is presented in Table 2. The broadest inhibition zone diameter was found in CS3 with 21.17 mm, while the narrowest one was in CS1 with 16.41 mm. The inhibited zone diameter of an edible film against the growth of *Salmonella* sp. can be seen in Figure 4.

E. coli and *Salmonella* sp. are Gram-negative bacteria, whose cell wall is thinner than that of Gram-positive bacteria. Chitosan damages Gram-negative bacteria more easily than Gram-positive bacteria because Gram-positive bacteria have thick cell walls consisting of more than 50% peptidoglycan and low lipid content (1-4%). The main function of cell walls is to provide a strong and rigid structure to maintain cell integrity. Therefore, thicker bacterial cell walls are harder to damage.

The result of the study indicates that casein-chitosan edible film has antimicrobial activity against E. coli and *Salmonella* sp. since bacteria cannot grow around the edible film. This supports the result of a study [28]. The antibacterial activity of chitosan against *Salmonella typhi*, another Gram-negative bacterium, is also documented [33]. Chitosan is attributed to pervasion throughout the microbial cell, thus disturbing the metabolism of E.coli [28]. The mechanism of inhibition action of chitosan against the growth of E. coli is well described [10]. Cell wall of bacterium is composed of peptidoglycan which is made from lipopolysaccharide and teicoic acid consisting of alcohol and phosphate. The presence of phosphate and alcohol means the cell wall has hydrophilic group. Thus, cell wall tends to have negative charge and is more polar in nature. When in contact with a bacterium, positively charged chitosan will interfere on the bacterium metabolism [33]. Based on these categories, the diameter of inhibition zone for E. coli growth as well as for Salmonella growth can be categorized as very strong.

![Figure 4. Edible film inhibition zone in *Salmonella* sp.](image)

### 4. Conclusion

The best casein-chitosan ratio was 1:4 (ml:ml). The greatest water vapor permeability with 0.1140 g.mm/m².h.kPa, film solubility with 32.809% and water activity with 0.709. Casein-chitosan edible films had strong antimicrobial activity against *S. aureus*, *L. bulgaricus*, *E. coli* and *Salmonella* sp.

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