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Optimization and characterization of biodegradable film based on glutinous flour/glycerol/chitosan/ZnO using Response Surface Methodology (RSM) - Central Composite Design (CCD)

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Abstract

Starch-based films are considered more competitive than petroleum because they are renewable, environmentally friendly, and easily degraded. The film in this study was fabricated from white glutinous flour, glycerol, chitosan, and ZnO through a starch gelatinization process. Chitosan content ranges from 2-4% (w/v), ZnO 4-8% of the dry weight of solid, and glycerol 15-45% of the dry weight of solids with a mass of white glutinous flour as much as 3 g was determined. Optimization and determination of running variables based on Central Composite Design. Response variables such as tensile strength, elongation, and water absorption were observed as important parameters in applying film as packaging materials. The Design Expert program recommended 2 g of chitosan: 8 % ZnO: 36.02% glycerol as the best composition in film fabrication, which aims to obtain maximum tensile strength and elongation, as well as minimum water absorption with the maximum desirability value (0.660). The predicted response values under optimal conditions by RSM were 3.68 MPa for tensile strength, 86.79% for elongation, and 268.09% for water absorption. The actual response has a tensile strength of 3.31 MPa, elongation of 83.5%, and water absorption of 320%. On average, a white glutinous flour/glycerol/chitosan/ZnO-based film required ± 45 days to degrade in the soil completely.

1. Introduction

Plastic is a synthetic or semisynthetic material consisting of a long chain of atoms bonded together. Polymer properties such as mechanical, optical or other properties depend highly on the material composition, size, arrangement, morphology, structure and molecular characteristics [1]. Plastics that are commonly used are fossil-based synthetic polymers, which are obtained from hydrocarbon derivatives and petroleum. The plastic produced is classified as difficult to decompose and takes a very long time, i.e. 300-500 years, to decompose completely [2]. This will certainly cause problems for the environment & organisms and the possibility of climate change.

The high use of plastic is due to the advantages of plastic compared to other materials, i.e. low production costs, lightweight, strong, durable, flexible, can be combined with other materials, and non-corrosive [1]. Economically, starch-based films are considered more uncomplicated and more affordable. Bacteria can degrade a biodegradable film made from starch by breaking polymer chains to produce water, carbon dioxide (CO_2), methane (CH_4) or other inorganic compounds that are not harmful to the environment. The starch film can prevent the transmission of moisture, oxygen, carbon dioxide, lipids and flavor components in food [3].

As an agricultural country, Indonesia has a relatively high agricultural productivity. Based on data from the Central Bureau of Statistics (Indonesia) [4], rice production in 2021 will increase by 1.14% (55.27 million tons of GKG) compared to 2020 (54.65 million tons of GKG). Glutinous is one of the rice varieties with a

reasonably high starch content, with 83.1% consisting of 1-2% amylose and 98-99% amylopectin. The time required for glutinous flour to gelatinize completely is faster with a lower temperature than other flours, requiring only 5.87 minutes at a gelatinization temperature of 67.47°C [5]. Haryanto and Saputri [6] compared the composition of tapioca flour and white glutinous flour film manufacture and showed that glutinous flour improves the film's mechanical properties. The source of starch used in this study came from white glutinous flour.

A plasticizing agent in the form of glycerol is added to increase the flexibility of the polymer because it can increase the distance between the molecules of the polymer. However, starch-glycerol-based biodegradable films still have drawbacks, such as low mechanical qualities and the inability to withstand heat, microorganisms, and water [7]. A starch-based biodegradable film needs additives to improve its mechanical properties to obtain the desired mechanical properties. In this study, chitosan and ZnO are combined as reinforcing agents. ZnO also acts as an antimicrobial compound that can reduce and inhibit the growth of microorganisms in packaged goods [8].

Chitosan is the second most abundant polysaccharide in the world after cellulose. It is found in the exoskeleton of invertebrates and some fungi in their cell walls, including milkfish scales, green clam shells, snail shells, crab shells, and shrimp shells. According to The Ministry of Marine Affairs and Fisheries Republic of Indonesia, shrimp is the mainstay of Indonesia's fishery sector export commodity by providing the largest contribution of total foreign exchange earnings for fishery exports, with the export volume of processed shrimp in 2018 reaching 145,226 tons. From the freezing process of shrimp for export, between 30-75% of the total weight of shrimp becomes shrimp shell waste and can be used as a source of chitosan [9]. The addition of chitosan mass composition to the film can increase the tensile strength, Young's modulus, and water resistance of biodegradable film due to the high bond intensity of C≡C bond in the sample so that the bond is more challenging to stretch or break.

ZnO is frequently utilized as a metal reinforcement since it is a piezoelectric ceramic with antibacterial characteristics [10]. Amni *et al.* [11] made biodegradable plastic from cassava starch by adding straw nanofibers and ZnO. The results indicated that ZnO produces biodegradable plastics with higher tensile strength and water resistance than straw nanofibers. The addition of 5% ZnO to the chitosan film showed an antimicrobial effect on *Salmonella typhimurium* bacteria. It is recommended that larger concentrations inhibit *Staphylococcus aureus* for medical and dental applications [12]. The antimicrobial-active films allow for an extended shelf life of the material due to bacterial growth. Therefore, the addition of antimicrobial substances to the film can reduce the use of chemical preservatives in food.

The use of the material in the industry and daily life depends on the material's mechanical properties. This property is one of the essential properties that indicate the strength of the material. High tensile and elongation values indicate a material with good mechanical properties [13]. Additionally, the application of biodegradable film as a packaging material needs to pay attention to the ability of the film to withstand the transfer of environmental water vapor into the polymer as determined by the water absorption test. Therefore, the tensile strength, elongation and water resistance of the fabricated biodegradable film were chosen as the desired response.

In most research, a design optimization necessitates time, resources, and a significant number of experiments. Consequently, Response Surface Methodology (RSM) is required to optimize the necessary factors to overcome these limitations and weaknesses. The application of Response Surface Methodology is carried out by inputting variables that have the potential to affect performance measures or product or process quality characteristics. This identification aims to reduce the number of variables used so that the experiment will be more efficient and require less testing [14]. The Central Composite Design and the Box Behnken are commonly used in constructing second-order models. The number of experiments on the box Behnken design is less than the central composite design since there are no axial/star runs in the box Behnken design [15].

The central composite design contains a combination of extreme factors, whereas the box Behnken does not examine the boundary area [16]. Therefore, the central composite design is considered more accurate and does not require a three-level factorial experiment in the operation of the quadratic model. Analysis of variance (ANOVA) was used to calculate the coefficient of determination, linear significance, fit, and interaction effect. The mean squared value was calculated by dividing the sum of the squares of each source of variation by its degrees of freedom. A 95% confidence level ($\propto = 0.05$) was used to determine statistical significance in all analyses [17].

This study used white glutinous flour to make biodegradable films by starch gelatinization method. The effect of levels and interactions of plasticizers (glycerol) and reinforcing agents (chitosan, ZnO) on white glutinous flour-based film was studied using Central Composite Design (CCD) combined with RSM. Optimal operating conditions were obtained and validated experimentally.

2. Experimental

This study was designed using Central Composite Design based on Response Surface Methodology (RSM) from statistical software Design Expert version 13 (Stat-Ease, USA) to analyze the data and determine the optimal composition in the preparation of biodegradable films. The range of variables was determined based on previous studies. de León *et al.* [18] used 1.5 g of chitosan in 50 mL of acetic acid, and Lian *et al.* [19] recommended a ZnO

concentration of 6% (w/w) of the dry weight of chitosan and starch. The research by Hu *et al.* [20] utilized a glycerol solution with a concentration of 30% of the dry weight of solids. The observed responses were tensile strength, elongation, and water absorption as essential parameters in applying biodegradable films as packaging materials. The independent variables can be seen in Table 1.

Table 1. Level of independent variables

	Variable	Limit code	
		Lower limit (-1)	Upper limit (+1)
A	Chitosan (gram)	1	2
B	ZnO (% w/w based on dry weight of chitosan and flour)	4	8
C	Glycerol (% v/w based on dry weight of solid)	15	45

2.1. Materials

Commercial white glutinous flour (Rose Brand) was used as a raw material for biodegradable film preparation. Chitosan from crab shells (viscosity and degree of deacetylation = 20-100 mPas and min 94%, respectively) with 98% purity was purchased at Phy Edumedia (Indonesia). ZnO powder 99.9% (pharmaceutical grade) was purchased at Heansa Kimia (Indonesia). The acetic acid reagent (99.8%) was an analytical reagent purchased from Merck (Indonesia). Distilled water and glycerol were obtained from the Integrated Laboratory, Diponegoro University.

2.2. Preparation of Chitosan/ZnO Solution

A number of masses of chitosan and ZnO (running variables from the Design Expert program) were dissolved in 50 mL of 2% (v/v) acetic acid under heating at 80°C and followed by stirring at 600 rpm for an hour until completely dissolved [12].

2.3. Preparation of White Glutinous Flour/ Glycerol

As much as 3 g of glutinous flour and glycerol (running variable from the Design Expert program) were dissolved in 50 mL of deionized water in a beaker glass to obtain a consistency of 6% w/v and heated at 80°C followed by stirring at a speed of 300 rpm for 15 minutes to form a glutinous flour/glycerol solution [19].

2.4. Preparation of Biodegradable Film from Glutinous Flour/Glycerol/Chitosan/ZnO

The resulting gelatinization solution of glutinous flour/glycerol was added with a solution of chitosan/ZnO. The mixture was heated at 90°C and stirred at 800 rpm for 30 minutes. The mixed solution was allowed to stand for 15 minutes to remove air bubbles. The final mixture was poured into an acrylic mold measuring 20 cm × 20 cm and dried for a week at room temperature, resulting in a film ready for analysis [19].

2.5. Starch Content Test (AOAC, 1970)

The sample weighed 0.5 to 1 g in a 250 mL beaker. The suspension was filtered using filter paper and washed with water until the filtrate volume reached 250 mL. The residue was washed with 200 mL of water and added 20 mL of 25% HCl. The beaker was covered with the reverse cooler and heated to a boil for 2.5 hours. The solution was then cooled and neutralized with 1 N NaOH and diluted to 250 mL. After neutral, the mixture was filtered using filter paper. The sugar content expressed as glucose from the filtrate obtained was calculated by multiplying the weight of glucose by a conversion factor of 0.9.

2.6. Amylose Content Test (IRRI, 1971)

Amylose will turn blue when it reacts with iodine compounds. The intensity of the blue color will vary depending on the amylose content in the material. A total of 40 mg of amylose was put into a test tube, and then 1 mL of 95% ethanol and 9 mL of 1 N NaOH were added. The test tubes containing the mixed solution were heated in boiling water for ± 10 minutes until the solution formed a gel. Pipette 1, 2, 3, 4 and 5 mL of each solution into a different 100 mL volumetric flask and add 0.2, 0.4, 0.6, 0.8, and 1 mL of 1 N acetic acid and 2 mL of iodine solution afterwards. Each mixture in a different test tube was added water to the mark and allowed to stand for 20 minutes. The intensity of the blue color formed was measured with a spectrophotometer at 625 nm.

2.7. Tensile Strength Test (ASTMD-882)

The tensile strength of biodegradable films was tested using a Universal Testing Machine at the Diponegoro University Integrated Laboratory with a tensile speed of 5mm/minute load cell scale of 4% of 100 kgf. The samples to be tested were cut according to the standard of 1 x 5 cm and conditioned in a climatic chamber with standard temperature and humidity (23±2°C) with 50±5% humidity for 40 hours. Both ends of the film are clamped on a modified mini-modified serrated tensile tester with a capacity of 100 kgf. The tensile strength can be calculated using equation (1) [21].

$$\text{Tensile strength} = \frac{F}{A} \quad (1)$$

where, F is the maximum voltage (kgf), and A is the surface area (cm^2).

2.8. Elongation at Break (ASTMD-882)

Elongation is the increase in the length of the material when tested with a tensile load to determine the elongation level of the material to be stretched just before breaking. The elongation test was carried out at the Diponegoro University Integrated Laboratory. The elongation test for biodegradable films is carried out by comparing the length increase that occurs with the length of the material before the tensile test is carried out. The elongation of biodegradable films can be determined using equation (2) [21].

$$\%\text{Elongation} = \frac{l-l_0}{l_0} \text{ x } 100\% \quad (2)$$

where, l is the elongation after breaking up (cm), and l_0 is the first length (cm).

2.9. Water Absorption (ASTMD 570-98)

The water absorption test aims to determine the water absorption ability of biodegradable film when immersed. The biodegradable film was cut to a size of 60×60 mm and dried in an oven at 105°C until a constant mass was obtained as the dry mass of the film (W_0). The biodegradable film was entirely soaked in distilled water at room temperature and should rest on the edge. At the end of 2 hours, the specimens should be removed from the water one at a time, dried by wiping the film with a dry cloth and weighed to the nearest 0.001 g immediately. The percentage of water absorption was calculated based on equation (3) [22].

$$\text{Water absorption} = \frac{W-W_0}{W_0} \text{x } 100\% \quad (3)$$

where, W and W_0 are the biodegradable film mass after and before soaking, respectively.

2.10. Antimicrobial Activity

The antimicrobial activity test was performed at the Central Java Province Health Laboratory and Medical Device Testing Center using the agar diffusion method to evaluate the antimicrobial activity of *Escherichia coli* (*E. coli*) (Gram-negative bacteria). A total of 13 g of nutrient broth was dissolved in 1000 mL of distilled water and then sterilized using an autoclave. One to two loops of microorganisms were cultured onto 50 mL agar culture media in different 100 mL Erlenmeyer flasks. The flasks were incubated using a rotary shaker at 37°C at a speed of 120 rpm for 24 hours. A total of 1 mL of bacterial culture was diluted in a 99 mL agar medium. A 10 mL of diluted media (~106 CFU/mL) was inoculated into a petri dish containing agar media and a 2 cm diameter biodegradable film test film that had been autoclaved previously. Petri dishes were incubated for 24 hours at 37°C under visible light (20 W). The zone of inhibition was measured to determine the antimicrobial activity of the composite film, and the average was recorded. The inhibition rate (%) can be calculated by equation (4).

$$\text{Inhibitory rate} = \frac{OD600_1 - OD600_2}{OD600_1} \text{ x } 100\% \quad (4)$$

2.11. Fourier Transform Infrared (FTIR) Analysis

Fourier Transform Infrared (FT-IR) is an infrared spectroscopy method equipped with a Fourier transform to analyze the spectrum resulting from the transmission of light passing through the sample. Light intensity was measured using a detector and compared with the intensity without the sample. Functional group analysis was conducted at the Integrated Laboratory of Diponegoro University with a transmittance mode at a resolution of 5 cm^{-1} ranging from 4000 to 450 cm^{-1}.

2.12. Soil Burial Test

The biodegradation test determines the level of film decomposition using the research method conducted by Armynah *et al.* [23] and Obasi *et al.* [24], with slight modification. The time of the sample to decompose can be seen from the reduction in the mass of each specimen planted in the soil. Initially, 1200 g of soil was placed in different pots with tiny holes perforated at the bottom and side of the container to increase air and water circulation. The sample was cut to 2×2 cm^2 and weighed until a constant mass (W_1) was obtained. Then the samples were buried in the soil at a depth of 3 cm from the surface and left for 31 days at room temperature. The soil surface was kept moist by spraying water every day. The percentage of weight loss can be calculated using equation (5).

$$\%\text{Weight loss} = \frac{W_1-W_2}{W_1} \text{ x } 100\% \quad (5)$$

where, W_1 and W_2 are the film weight before and after the biodegradation test, respectively.

3. Results and Discussion

3.1. Flour Composition

The content of amylopectin affects the stability of the films because amylopectin has a very high molecular weight that reduces the mobility of the polymer chains and produces gels with high viscosity [25]. Meanwhile, starch with high amylose content will produce a flexible and strong film. The smaller the amylose content or, the higher the amylopectin content, the more sticky the gel produced. However, the mechanical properties of the film will increase when the amylose content is increased. Glutinous flour used as raw material for making a biodegradable film in this study was analyzed for starch composition to determine the content of starch, amylose and amylopectin at Chem-Mix Pratama Laboratory, Yogyakarta.

Table 2. Composition of glutinous flour

Flour	Starch (%)	Amylose (%)	Amylopectin (%)
White glutinous	89.15	5.65	83.50

As shown in Table 2, the content of glutinous rice flour used in this study was very high at 89.15%, consisting of amylose and amylopectin. The high amylopectin content (83.50%) in the material will produce a more stable film because it has a very high molecular weight, reducing the polymer chains' mobility and producing gels with high viscosity [25]. Meanwhile, low amylose content (5.65%) will produce films with low mechanical strength. The structure of amylose allows the formation of hydrogen bonds between its constituent glucose molecules. During heating, it can form a three-dimensional network that can trap water and stop the flow of liquid around it so that it undergoes a process of particle orientation. The interhelical and intrahelical hydrogen bonds formed led to the formation of a hydrophobic structure with low solubility [26].

3.2. Optimization of Experimental Design

The starch film was produced through the gelatinization process of glutinous flour, which is the irreversible process of destroying the crystal structure of starch granules. The gelatinization process involves granular swelling, liquefaction of native crystals and dissolution of molecules. The initial stage of gelatinization occurs when the granules interact with water, accompanied by an increase in temperature [27]. Amylose and amylopectin partially separated during

gelatinization due to incompatibility caused by an increase in water stress and temperature. All amylose diffuses out of the granules and leaves only amylopectin [28]. When starch granules are heated at 80°C, hydrogen bonds are broken, and water molecules become bound to the hydroxyl groups in the starch molecules, resulting in greater swelling and dissolution of the crystals. The granules will swell and break so that the semi-crystalline arrangement is gone, and the short branched chains (amylose) will form gel-balls at the end of the chain in the shape of strands of rope-like layers. This network compresses water and increases the viscosity of the mixture to form a thick solution [27].

Table 3. Central Composite Design experiment results

Factor			Response		
Chitosan (A)	ZnO (B)	Glycerol (C)	Tensile strength (MPa)	Elongation (%)	Water absorption (%)
1.5	6	4.77	4.16	9.7	217
1.5	6	55.23	0.5	135	810
2	8	45	3.13	104	412.5
1.5	6	30	1.96	44.7	225
1.5	6	30	1.16	63.3	228
1.5	6	30	1.56	83.5	200
1	8	15	2.29	55.5	90
1	4	45	0.49	70	500
1.5	6	30	1.39	81.3	240
1.5	9.36	30	2.95	87.3	83.33
1.5	6	30	1.56	86.3	280
2.34	6	30	5.13	88	465
2	4	15	3.44	31.3	407
2	4	45	1.01	147	600
0.66	6	30	0.52	53.3	118.18
1	4	15	2.33	22.2	100
1	8	45	0.8	94	399
2	8	15	4.72	18.2	120
1.5	2.64	30	0.89	64.5	412.5
1.5	6	30	1.27	81.3	342.86

The effect of chitosan and ZnO as a reinforcing agent and glycerol as a plasticizer on the mechanical properties of the biodegradable film was investigated based on Central Composite Design to optimize the design process and product formulation. The response measured in film mechanical properties, including tensile strength, elongation and water absorption, is related to the essential parameters in applying biodegradable film as packaging material. A total of 20 trials were used for RSM modeling, and the order of trials was arranged randomly. The experimental results are shown in Table 3.

3.3. Statistical Analysis

Design Expert version 13 was used to design the optimal conditions for independent variables by providing model summary statistics and variance ANOVA analysis in assessing the model's validity and the suitability of the polynomial equation model. Polynomial equations must be applied to study the connection between the dependent and independent variables. This mathematical model can describe the response surface linearly, the interaction of two factors (2FI), squared and cubic. RSM will predict the most suitable mathematical model to describe the relationship between responses and factors without further complicated and time-consuming numerical simulations [29]. The empirical relationship between the observed response and factor effects is expressed by a second-order polynomial equation (6), (7), and (8).

$$\text{Tensile strength} = 1.49 + 1.04\,A + 0.52\,B - 0.99\,C + 0.39\,AB - 0.09\,AC + 0.15\,BC + 0.45\,A^2 + 0.13\,B^2 + 0.27\,C^2 \quad (6)$$

$$\text{Elongation} = 71.02 + 8.58\,A + 2.90\,B + 36.50\,C - 14.18\,AB + 14.40\,AC - 4.90\,BC \quad (7)$$

$$\text{Water absorption} = 253.56 + 75.70\,A - 83.41\,B + 160.49\,C - 45.44\,AB - 27.94\,AC + 1.06\,BC + 7.78\,A^2 - 7.67\,B^2 + 86.23\,C^2 \quad (8)$$

Code equations are useful for identifying the relative impact of factors by comparing factor coefficients. The positive and negative signs of the regression coefficients represent each variable's synergistic and antagonistic effects on the response. The model coefficients are represented by constant terms A, B and C (linear coefficients for the independent variables), AB, AC and BC (coefficients of interactive terms), and A^2, B^2 and C^2 (coefficients of quadratic terms) [30].

3.4. The Effect of Chitosan, ZnO and Glycerol Concentration on Tensile Strength

Analysis of variance (ANOVA) explains the interaction between the levels of chitosan, ZnO and glycerol with the tensile strength response can be described through a quadratic model, as shown in Table 4. The significance of each model parameter was determined based on the F-value and p-value. Factors that have a significant influence on the tensile strength response in this study are chitosan (A), ZnO (B), glycerol (C), chitosan interaction with ZnO (AB), chitosan quadratic factor (A^2) and glycerol quadratic factor (C^2).

Table 4. Results of quadratic model ANOVA for tensile strength response

Source	Sum of Squares	df	Mean Square	F-value	p-value	Statement
Model	36.86	9	4.10	23.50	<0.0001	Significant
Chitosan (A)	14.65	1	14.65	84.04	<0.0001	Significant
ZnO (B)	3.73	1	3.73	21.38	0.0009	Significant
Glycerol (C)	13.36	1	13.36	76.63	<0.0001	Significant
AB	1.22	1	1.22	7.03	0.0243	Significant
AC	0.0595	1	0.0595	0.3415	0.5719	Non-significant
BC	0.1770	1	0.1770	1.02	0.3373	Non-significant
A^2	2.86	1	2.86	16.43	0.0023	Significant
B^2	0.2283	1	0.2283	1.31	0.2791	Non-significant
C^2	1.06	1	1.06	6.06	0.0335	Significant
Residual	1.74	10	0.1743	-	-	-
Lack of fit	1.35	5	0.2690	3.38	0.1036	Non-significant
Pure error	0.3977	5	0.0795	-	-	-
Cor Total	38.60	19	-	-	-	-

Based on Table 5, the value of R^2 explains that the independent variable has the ability of 95.48% to provide the required information and simultaneously affects the tensile strength. The difference between adjusted R^2 and predicted R^2 values less than 0.2 explains that the model is a good fit between the actual data and predictions. Adequate precision obtained at 17.25 indicates an adequate signal so that the model can be applied to navigate the design space. The coefficient of variance (C.V.%) is an estimate of the ratio of the standard error to the mean of the observed responses and is determined based on the reproducibility of the model. The C.V.% value in this study was 20.24 (C.V.% >10), so the model can be used and shows excellent precision and consistency from the experimental values obtained. Graphical regression equations for optimization of reaction conditions are depicted through a three-dimensional response surface as an approach that describes the condition of the reaction system. The results of the interaction between factors and responses are presented in Figure 1.

Figure 1(a, b) shows the effect of the interaction of chitosan and ZnO on the tensile strength of biodegradable films. The film's tensile strength increased with the increasing concentration of chitosan and ZnO in the polymer. Increasing the concentration of ZnO will increase hydrogen bonds in the film, resulting the biodegradable films with higher tensile strength. However, an excessive concentration of ZnO will reduce the tensile strength of biodegradable films. This is because the density and improper particle distribution at high concentrations will reduce cross-connection in the film, increase chain mobility, and cause damage to the polymer network structure [31].

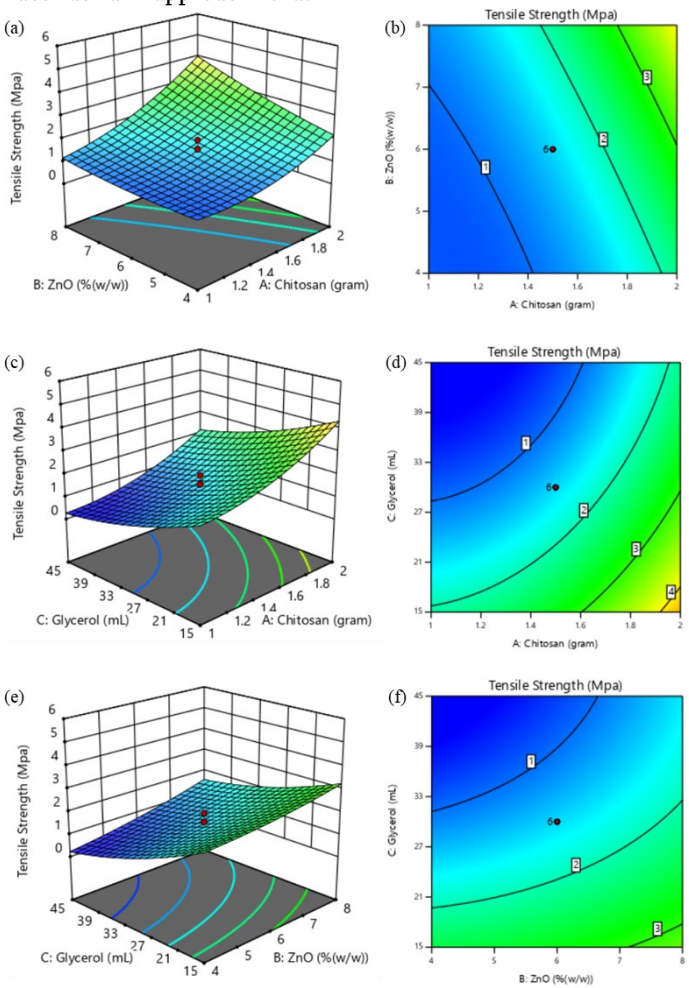

Figure 1. Three-dimensional tensile strength response surface plots and contour plots showing the effects of (a) and (b) chitosan and ZnO; (c) and (d) chitosan and glycerol; (e) and (f) glycerol and ZnO

Meanwhile, adding chitosan to manufacture biodegradable film will also increase hydrogen bonds between chitosan and polymers, thereby increasing the film's density and causing the intermolecular bonds to become stronger and hard to degrade [32]. Chitosan is a cationic polymer that effectively binds heavy metal ions and cations from organic substances since it contains amino and hydroxyl groups. The interaction between metal cations and chitosan will occur by forming a chelate ring between chitosan and ZnO by the N atom in the amino group and O in the hydroxyl group [33]. Afterwards, the chitosan and ZnO groups will capture electrons from the hydroxyl group (-OH) in polymers. The uniform dispersion of ZnO and chitosan in the glutinous flour polymer matrix builds strong interactions through ionic bonds, thereby increasing the adhesion properties of the film [11]. Therefore, the tensile strength response will increase as the interaction between chitosan and ZnO increases.

Table 5. Fit statistical results of ANOVA for tensile strength response.

Std. Dev	0.4175	R^2	0.9548
Mean	2.06	Adjusted R^2	0.9142
C.V.%	20.24	Predicted R^2	0.7193
		Adeq Precision	17.2553

Another factor influencing the tensile strength response is glycerol, as shown in Figure 1(c, e). The tensile strength response will decrease as the glycerol concentration increases. This is due to the weakening of the hydrogen bonds that were initially formed between the amylose and amylopectin molecules due to the addition of glycerol [17]. Low-molecular-weight glycerol as plasticizers has several roles, such as intercalating between polymer chains, breaking hydrogen bonds, reducing intermolecular forces, increasing polymer network mobility, flexibility, glass transition temperature and water vapor and gas permeability. Glycerol molecules will diffuse into the polymer chain and result in the plasticization of the intra-bundle infrastructure [34].

3.5. Effect of Chitosan, ZnO, and Glycerol Concentration on Elongation

Elongation at break (EAB) is the ratio between the length of a material before and after a tensile test due to the applied load or force [35]. The ANOVA results, as listed in Table 6, show that the relationship between factors and elongation responses is explained through the 2FI mathematical model (two-factor interaction). Based on the significance of the model and lack of fit, it shows that the model used in this study has the appropriate response data with the model. Factors that had a significant influence on the tensile strength response in this study were chitosan (A), glycerol (C), the interaction of chitosan with ZnO (AB), and the interaction of chitosan with glycerol (AC).

Table 6. Results of model 2FI ANOVA for elongation response

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Statement
Model	22776.22	6	3796.04	28.66	<0.0001	Significant
Chitosan (A)	1005.07	1	1005.07	7.59	0.0164	Significant
ZnO (B)	114.51	1	114.51	0.8645	0.3694	Non-significant
Glycerol (C)	18198.25	1	18198.25	137.40	<0.0001	Significant
AB	1607.44	1	1607.44	12.14	0.0040	Significant
AC	1658.88	1	1658.88	12.52	0.0036	Significant
BC	192.08	1	192.08	1.45	0.2500	Non-significant
Residual	1721.81	13	132.45	-	-	-
Lack of fit	402.87	8	50.36	0.1909	0.9802	Non-significant
Pure error	1318.94	5	263.79	-	-	-
Cor Total	24498.03	19	-	-	-	-

Based on Table 7, the coefficient of determination R^2 of the elongation response in this experiment is classified as very high (0.75-1.00) and close to 1. Furthermore, the difference values of adjusted R^2 and predicted less than 0.2. Thus, the model can be relied upon to predict the elongation properties of the film. The adequate precision obtained is 20.13 (greater than 4), indicating an adequate signal so the model can navigate the design space. The C.V.% value in the elongation response is 16.20 (C.V.% >10), then the model can be used and shows excellent precision and consistency from the experimental values obtained. The graphic of the regression equation for the optimization of reaction conditions is depicted through a response that describes the conditions of the reaction system, which is presented in Figure 2.

Table 7. Fit statistical results of ANOVA for elongation response

Std. Dev	11.51	R^2	0.9297
Mean	71.02	Adjusted R^2	0.8973
C.V.%	16.20	Predicted R^2	0.8587
		Adeq Precision	20.1297

Increasing the concentration of chitosan and ZnO in this study will increase the concentration of added glycerol because it is based on the dry weight of the solids used. Based on Figure 2, the glycerol content provides a synergistic effect on the elongation response. The addition of glycerol will reduce the hydrogen bonds in the starch and chitosan molecules, making the distance between the polymer molecules tenuous. The hydroxyl group in glycerol will reduce the intermolecular bonds in the polymer due to the formation of hydrogen bonds between glycerol and polymer molecules, resulting in reduced intermolecular strength of the polymer and increasing the film's flexibility. However, the concentration of glycerol used as a plasticizer must be controlled because if it exceeds the compatibility limit, it will have a negative impact on the film produced, creating a heterogeneous system and a film that is readily ripped due to the film's excessive elasticity [36].

The effect of the interaction of chitosan and ZnO on the elongation response of biodegradable film based on glutinous flour is shown in Figure 2(a). The interaction between chitosan and ZnO in the polymer matrix has a detrimental impact on the elongation value of the film, confirmed by the value of the constant AB in equation (7). This is because the interaction between chitosan and ZnO forms a strong interfacial bond and increases hydrogen bonds in the polymer. The addition of chitosan and ZnO reduced the free space between chains and increased the film density. Therefore, the interaction of chitosan and ZnO increases the stiffness and decreases the flexibility of the polymer film [37].

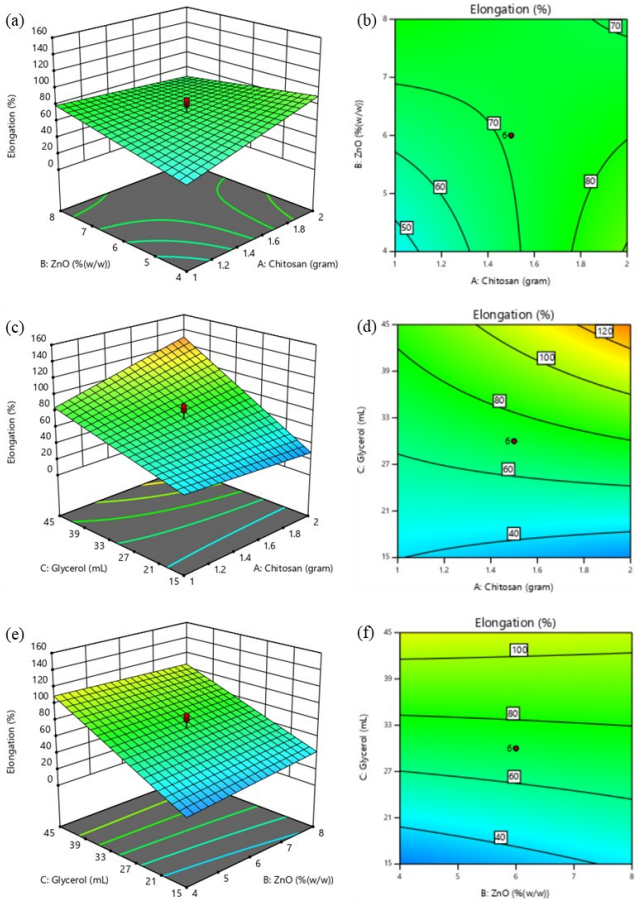

Figure 2. Three-dimensional tensile strength response surface plots and contour plots showing the effects of (a) and (b) chitosan and ZnO; (c) and (d) chitosan and glycerol; (e) and (f) glycerol and ZnO

The effect of the interaction of chitosan and ZnO on the elongation response of biodegradable film based on glutinous flour is shown in Figure 2(a). The interaction between chitosan and ZnO in the polymer matrix has a detrimental impact on the elongation value of the film, confirmed by the value of the constant AB in equation (7). This is because the interaction between chitosan and ZnO forms a strong interfacial bond and increases hydrogen bonds in the polymer. The addition of chitosan and ZnO reduced the free space between chains and increased the film density. Therefore, the interaction of chitosan and ZnO increases the stiffness and decreases the flexibility of the polymer film [37].

3.6. Effect of Chitosan, ZnO, and Glycerol Concentration on Water Absorption

Water absorption is a standard measure used to determine the relative rate of water absorption by the film when immersed. This test method for the rate of water absorption has two chief functions. First, to serve as a guide to the effects of exposure to water or humid conditions on electrical insulation resistance, dielectric losses, mechanical strength, appearance, and dimensions. Second, as a control test on the uniformity of a product. The quadratic model on the water absorption response was selected based on regression analysis using ANOVA Table 8. A significant model and an insignificant lack of fit indicate the adequacy of the model used to build the relationship between factors and responses. The independent variables include chitosan (A), ZnO (B), glycerol (C), the interaction of chitosan with ZnO (AB), and the quadratic factor of glycerol (C^2) are significant parameters for the water absorption response.

Table 8. Results of quadratic model ANOVA for water absorption response

Source	Sum of Squares	df	Mean Square	F-value	p-value	Statement
Model	6.588E+05	9	73199.94	22.88	<0.0001	Significant
Chitosan (A)	78253.27	1	78253.27	24.46	0.0006	Significant
ZnO (B)	95009.17	1	95009.17	29.70	0.0003	Significant
Glycerol (C)	3.518E+05	1	3.518E+05	109.96	<0.0001	Significant
AB	16516.53	1	16516.53	5.16	0.0464	Significant
AC	6244.03	1	6244.03	1.95	0.1926	Non-significant
BC	9.03	1	9.03	0.0028	0.9587	Non-significant
A^2	871.34	1	871.34	0.2724	0.6131	Non-significant
B^2	846.79	1	846.79	0.2647	0.6181	Non-significant
C^2	1.072E+05	1	1.072E+05	33.50	0.0002	Significant
Residual	31990.79	10	3199.08	-	-	-
Lack of fit	18801.25	5	3760.25	1.43	0.3534	Non-significant
Pure error	13189.54	5	2637.91	-	-	-
Cor Total	6.908E+05	19	-	-	-	-

Table 9. Fit statistical results of ANOVA for water absorption response

Std. Dev	56.56	R^2	0.9537
Mean	312.52	Adjusted R^2	0.9120
C.V.%	18.10	Predicted R^2	0.7556
		Adeq Precision	18.2684

Based on Table 9, the coefficient of determination R^2 is classified as very high, implying that this model is reliable and can explain the range of variables studied well. In addition, the difference in value between adjusted R^2 and predicted R^2 is less than 0.2, so the model used has a good fit between the actual and predicted data. Adequate precision (more than 4) in measuring the signal-to-noise ratio so the model can navigate the design space. The C.V.% value in this study was 18.10 (C.V.% >10), indicating a high level of precision and good reliability of experimental values. The three-dimensional (3D) response surface in Figure 3 explains the reciprocal interaction between the independent variables and the water absorption response.

Water absorption is one of the essential film parameters since the presence of water is crucial for the growth of microorganisms and physicochemical reactions in products, particularly food. This parameter was studied to determine the effect of the concentration of chitosan, ZnO and glycerol on the resistance properties of a biodegradable film based on glutinous flour to water vapor. The transfer of water vapor from the film depends on the solubility and the permeability of water molecules. Chitosan films show a high degree of solubility due to their hydrophilic properties. Chitosan-based films are quite susceptible to water because the amino and hydroxyl groups on the chitosan backbone tend to interact with water. This is confirmed by the positive value of the constant in the chitosan variable equation (8).

However, the solubility of the film decreased with the addition of ZnO due to the hydrophobic nature of the ZnO particles causing the binding of water molecules to the starch to decrease by reducing the free hydrogen groups. The intermolecular interactions between the polymer matrix and ZnO will create a structure with fewer pores, reducing the rate of penetration of water molecules into the film [18]. In addition, the interaction between chitosan and ZnO tends to reduce the free space between the polymer chains, thereby reducing the diffusivity, transmission rate and penetration of water vapor through the matrix due to the formation of winding paths through which water molecules will pass. The better the distribution of chitosan and ZnO in the polymer matrix, the higher the ability to inhibit the film against water vapor and gas [38].

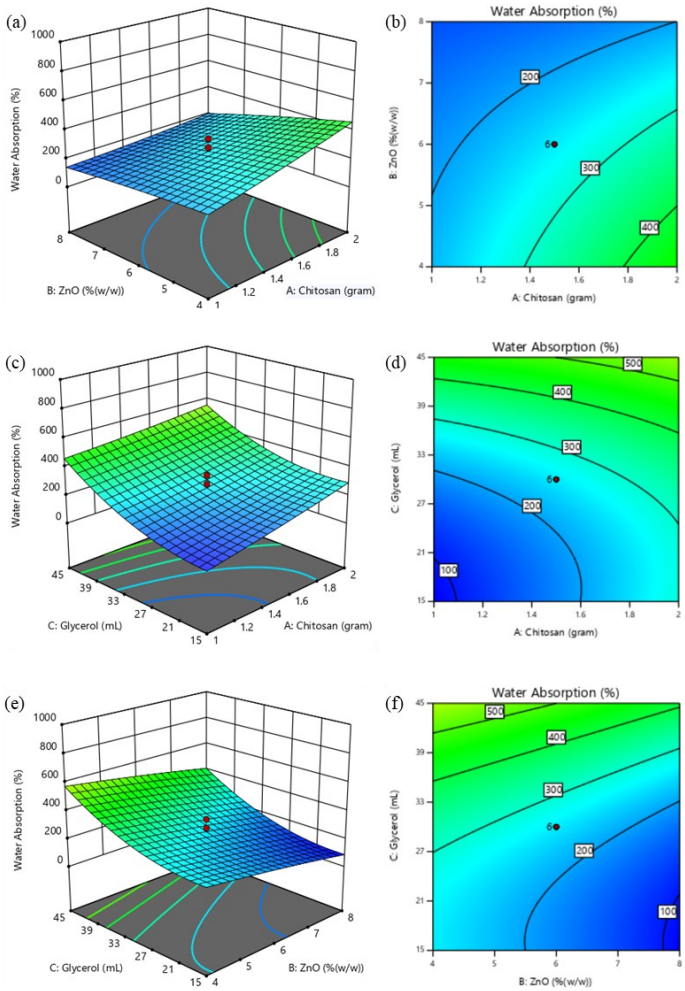

Figure 3. Three-dimensional tensile strength response surface plots and contour plots showing the effects of (a) chitosan and ZnO; (b) chitosan and glycerol; (c) glycerol and ZnO

Higher permeability was obtained after the addition of glycerol in glutinous flour. The inclusion of glycerol molecules weakens the hydrogen bonds in the starch and also due to the increased inter-chain distance due to the entry of glycerol molecules, which increases the diffusability of water vapor through the film resulting in an accelerated rate of water vapor transmission. The rate of water vapor transmission increases with the increase in glycerol due to the hydrophilic effect and lower amylose content in starch. The interaction of glycerol and acetic acid between the polymer chains also causes an increase in the distance between the chains, thereby increasing the diffusability of water vapor through the film [31].

3.7. Optimization

Numerical optimization predicts the optimal level of the independent variable to achieve the desired value in all responses. Factors were set within the range to obtain maximum tensile and elongation strength responses. On the other hand, water absorption responses were set to a minimum. The optimum combination of factors (0.660) was obtained by applying the desirability function method. Optimum conditions were obtained at 2 g of chitosan, 8% ZnO concentration, and 36.02% glycerol concentration. Under optimal conditions, the response values predicted by RSM were 3.68 MPa for tensile strength, 86.79% for elongation, and 268.09% for water absorption. The actual response had a tensile strength of 3.31 MPa, elongation of 83.5%, and water absorption of 320% (Table 10). The verification results were in the 95% prediction interval range, so it can be concluded that the solution recommended by the program Design-Expert is adequate.

Table 10. Response value predicted by software and measured experimentally

Analysis	Unit	Predicted	Measured	95% PI Low	95% PI High
Tensile strength	MPa	3.68	3.31	2.58	4.78
Elongation	%	86.79	83.5	57.64	115.92
Water Absorption	%	268.09	320	118.75	417.32

3.8. Antimicrobial Activity Results

Antibacterial activity testing was conducted to determine the film's ability to inhibit bacterial growth. The antimicrobial-active film allows for the extension of the shelf life of foods without the addition of excessive chemical preservatives during food processing. The antimicrobial activity test using the agar diffusion method to evaluate the antimicrobial activity of *E. coli* on the optimum film without ZnO and with the addition of ZnO is shown in Table 11.

Table 11. Results of inhibitory activity against *E. coli*

Sample	Inhibition Zone (mm)
Optimum film (without ZnO)	0
Optimum film (with ZnO)	35

The biodegradable film without additional ZnO has no inhibitory activity against *E.coli*. Meanwhile, a biodegradable film with additional ZnO showed an inhibitory activity with an inhibition zone of 35 mm. The same thing was reported by Perelshtein *et al.* [39], where the chitosan film had antimicrobial activity against Gram-positive bacteria but had little effect on Gram-negative bacteria. This is because gram-negative bacteria, especially *E.coli*, have a double outer membrane consisting of lipopolysaccharide and peptidoglycan, so they are less susceptible to surface disturbances than gram-positive bacteria, which only have a single membrane.

In addition, Li *et al.* [37] reported that adding nano-ZnO to the chitosan composite film significantly increased its antibacterial activity. Chitosan/ZnO film has an abrasive surface texture that can affect the antibacterial mechanism sequentially by damaging the bacterial membrane so that it has the potential to provide a synergistic effect in the composite film [39]. The antibacterial activity of glutinous flour/chitosan/glycerol/ZnO films can be attributed to electrostatic interactions between positively charged amino groups and negatively charged bacterial cell walls, leading to forming pores. Intracellular material can pass through the formed pores, causing physiological changes and bacterial cell death due to the penetration of Zn^{2+} ions and oxidative stress generated by ZnO in the bacterial cell wall [40]. Regarding the safety of using ZnO, several studies provide evidence of cytocompatibility. The study reported that there is no adverse effect on platelets adhering to the surface of Zn alloys, so the addition of ZnO in the synthesis of a biodegradable film can be recommended as a packaging or medical material at low concentrations [12].

3.9. Fourier Transform Infrared (FTIR) Analysis

FTIR analysis was employed to identify the functional groups present in the film based on the typical spectrum produced. The results of the FTIR analysis of glutinous flour are shown in Figure 4. As shown in Figure 4, the biodegradable film showed a strong absorption peak at 3290.52 cm^{-1}, attributed to the hydrogen bonds (O-H) of carbohydrates and protein. The peak was found at 2110.71cm^{-1} due to C=C conjugated, which has a strong relationship with mechanical strain and bioplastic degradation [12]. Meanwhile, the peak at 1924.63cm^{-1} explains C=C=C stretching and C-H bending in the film. The absorption band indicates the presence of glycerol and its role in forming hydrogen bonds that cause cross-linking in the biopolymer chain [41]. The absorption region of 2932.68cm^{-1} is associated with the C-H bond. It represents the deformation modes of the C-H_2 and C-H_3 bonds, where the intensity was modified by the insertion of glycerol into the film [41]. Chitosan residues that were not deacetylated in the form of amide-I, amide-II, and amide-III also appeared in each absorption area of 1411.36cm^{-1}, 1569.41cm^{-1}, and 1334.83cm^{-1}, respectively [12]. The absorption peak for amide-I comes from the C=O strain vibration in –$NHCOCH_3$ [42], while the amide-II comes from the N-H bond in the primary amine group (NH_2/-NH_3^+) [43]. Gamboa-Solana *et al.* [12] reported that 1151.02 cm^{-1} and 1020.07 cm^{-1} are absorption regions for Zn-O and N-Zn bonds.

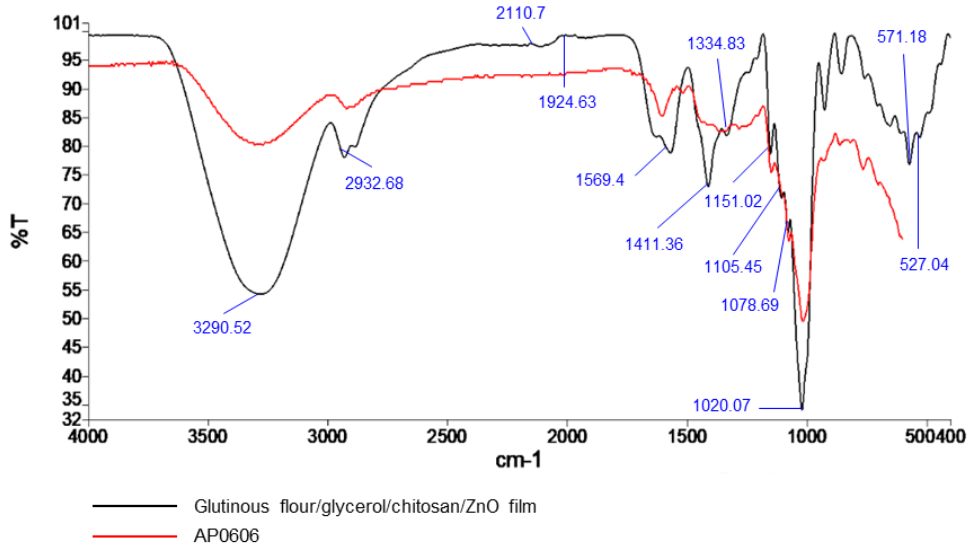

Figure 4. Result of FTIR analysis for biodegradable film

In addition, Dobrucka and Długaszewska [44] explained that the absorption region of 400cm^{-1} to 600cm^{-1} can be associated with the vibration of the Zn-O-Zn bond. The band observed at 1105.45cm^{-1} is due to the C-O-C asymmetric vibration of the glycosidic bond and 1078.69cm^{-1} to the C-O and C-N symmetrical and asymmetric tensile vibrations, which indicates the saccharide structure of the starch and chitosan-derived films. The absorption regions of 900 to 800 cm^{-1} are attributed to the presence of C=C, C=N, and C-H functional groups in the film [31]. The FTIR test results show a functional group of C=O carbonyl and C-O ester, indicating the degradability of the synthesized plastic because the functional groups O-H, C=O carbonyl and C-O ester are hydrophilic groups, water molecules can allow microorganisms in the surrounding environment to enter the plastic matrix. Increased tensile strength and biodegradability are caused by strong bonds between constituents, as shown by the existence of C=C and C-H bond absorption [43]. The FTIR spectrum analysis indicates that the optimum film sample has confirmed the structure of the chitosan, starch, glycerol and ZnO materials.

3.10. Film Biodegradability in Soil

The biodegradation process is the breakdown of polymeric organic compounds by microorganisms (bacteria, fungi, and algae) with the aid of intracellular and extracellular enzymes (endo and exoenzymes). This breakdown causes chemical reactions, such as cutting polymer chains and oxidation and producing water, carbon dioxide, biomass, and other primary products of biotic decomposition [45]. A starch-based biodegradable film can be degraded by *Pseudomonas* and *Bacillus* bacteria by breaking polymer chains into monomers. This test helps determine the sample's degradation rate and predict the time required for the sample to be decomposed by microorganisms in the soil [46]. Several samples were selected to represent the entire sample in determining its biodegradability in the soil, as shown in Table 12.

Table 12. Results of the biodegradation test

Sample	Chitosan (gram)	ZnO (%)	Glycerol (%)	Weight loss after 30 days (%)
R1	1.5	6	4.77	37%
R2	1.5	6	55.23	99%
R4	1.5	6	30	63%
R10	1.5	9.36	30	52%
R12	2.34	6	30	68%
R15	0.66	6	30	76%
R19	1.5	2.64	30	82%

The initial degradation of film in the soil is driven by energy from the sun. Soil temperature increases when exposed to sunlight energy and decreases when temperature drops and humidity increases, putting pressure on the film. The film absorbs an abundance of oxygen and H_2O during the night. The content of H_2O and O_2 will break bonds when it gets energy from the sun and produce OH^- and O^- ions. When energy from the sun enters the sample, ZnO in the film will produce an electron pair (e^-)-hole (h^+). (h^+) will react with H_2O molecules to produce OH^- and H^+, while (e^-) will react with O_2 to produce O^- and O_2. These ions are more reactive and attach to the hydrogen bonds of starch and chitosan and continue to break the bonds of other atoms producing a decrease in molecular weight, strength, and weight and ultimately cutting the film into small pieces. The mechanism of biodegradation involves penetration of water molecules, breaking of strong covalent bonds, and breakdown of hemicellulose & cellulose by the action of microorganisms. These processes will change the film's chemical, mechanical, and physical properties to produce final products of CH_2, CO_2, H_2O, biomass and energy [18]. On average, a biodegradable film based on white glutinous flour/glycerol/chitosan/ZnO requires ± 45 days to degrade in the soil completely.

In the manufacture of biodegradable plastics, glycerol has an important role. Starch is a natural polymer in the form of granules that cannot be processed into thermoplastic materials because of the strong intermolecular and intramolecular hydrogen bonds.

However, the hydrogen bonds can be broken and starch can be processed into biodegradable polymers in the presence of water and a plasticizer (glycerol) [47]. The interaction of glycerol with the polymer matrix will reduce the intermolecular bonds in the polymer due to the formation of hydrogen bonds between glycerol so that the distance between the biopolymer molecules becomes tenuous. This condition reduces the intermolecular strength of the polymer so that it is more easily degraded [36]. The presence of chitosan and ZnO in the polymer increases the intermolecular and intramolecular strength of hydrogen bonds by capturing electrons from the hydroxyl group (OH) on the polymer so that it will increase the polymer's resistance to mechanical treatment [11]. In addition, the degradation value decreases with increasing concentrations of ZnO due to its hydrophobic nature, thus decreasing the water absorption from the soil into the bioplastic matrix. ZnO also acts as a nanofiller that functions to close the pores of the starch bioplastic matrix so that bioplastics containing ZnO tend to be stiffer.

4. Conclusion

The biodegradable film of glutinous flour/glycerol/chitosan/ZnO has been successfully made via the starch gelatinization method. The fabrication process used Central Composite Design through Design Expert version 13.0 by evaluating the composition of chitosan, ZnO, and glycerol as independent variables affecting the film's mechanical properties, such as tensile strength, elongation and water absorption. After optimization, the Design Expert program recommended 2 g of chitosan: 8 % ZnO: 36.02% glycerol as the best composition in film fabrication, which aims to obtain maximum tensile strength and elongation, as well as minimum water absorption with the maximum desired value (0.660). The predicted response values under optimal conditions by RSM were 3.68 MPa for tensile strength, 86.79% for elongation, and 268.09% for water absorption. The actual response has a tensile strength of 3.31 MPa, elongation of 83.5%, and water absorption of 320%. On average, a biodegradable film based on white glutinous flour/glycerol/chitosan/ZnO requires ± 45 days to degrade in the soil completely.

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