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Original Research Article

Stability Study and Antifungal Activity of Chitosan Films from Shrimp Shells against *Colletotrichum gloeosporioides*

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ABSTRACT

Chitosan, a modified product of chitin has found application in various fields such as in foods, cosmetics, agricultural products, and pharmaceuticals. This study aim to evaluate the stability and the antifungal activity of chitosan films produced from shrimp shells. Chitosan films with various degrees of deacetylation (70%, 80%, and 90%) were produced from shrimp shells by solution casting and solvent evaporation method. The stability of the chitosan films was evaluated under a controlled environmental conditions over a 35 days period. The key stability parameters monitored include; tensile strenght, elongation at break, elastic modulus, and water vapour permeability. The antifungal activity of the films was investigated against *Colletotrichum gloeosporioides* using the disc diffusion method. After 35 days, the mechanical properties (tensile strenght, elongation at break, and elastic modulus), and water vapour permeability of the chitosan films remained largely unchanged, indicating sustained stability over time. The chitosan films at the three deacetylation levels (70%, 80%, and 90%) exhibited antifungal activity against *Colletotrichum gloeosporioides*, with chitosan film with 90% degree of deacetylation showing the highest antifungal activity. From the results of the study, chitosan films at three degrees of deacetylation (70%, 80%, and 90%) have good stability under the storage conditions tested. They are highly active against *Colletotrichum gloeosporioides*, thus could be used as protective films for food preservation.

Keywords: Antifungal, Chitosan film, *Colletotrichum gloeosporioides*, Stability.

Introduction

Chitosan is a modified product of chitin, formed by removing acetyl groups from chitin molecules through a process called deacetylation.[1,2] Chitosan dissolves well in acidic environments, possesses film-forming capabilities and exhibits optical properties.[3] It finds applications in various fields such as cosmetics, food, pharmaceuticals, chemical industries, and healthcare. Chitosan was first discovered by Rouget in 1859. It is an amino polysaccharide formed by removing acetyl groups from chitin through concentrated alkali processing. Although chitosan, chitin, and cellulose have similar structural formulas, they differ in properties. Chitin only dissolves in a few solvents, such as dimethylacetamide/Lithium chloride mixture, whereas chitosan easily dissolves in organic acids (usually acetic acid), making it more versatile than chitin.[4,5] Chitosan, a naturally sourced material, is non-toxic and safe. It exhibits high biocompatibility with the human body and has the ability to undergo biological self-degradation.[6]

The antibacterial property of chitosan derived from shrimp shells against *E. coli* is demonstrated through its ability to inhibit bacterial growth. The mechanism of this inhibition is attributed to the binding of chitosan polymer chains with metal ions on the bacterial surface, altering the permeability of the cell membrane.

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Introduction of chitosan into the microenvironment causes the neutralization of negative charges on the bacterial cell surface, alters cell membrane permeability, and disrupt cellular integrity.[7] The effectiveness of this antibacterial property depends on the molecular weight of chitosan and bacterial species. For Gram-positive bacteria, chitosan with a molecular weight of 470 daltons was shown to have a significant impact on various species except for *Lactobacillus* sp., whereas, for Gram-negative bacteria, chitosan with a molecular weight of 1106 daltons was effective against a few species. This indicates that chitosan has a stronger influence on Gram-positive bacteria such as *Listeria monocytogenes*, *Bacillus megaterium*, *B. cereus*, *Staphylococcus aureus* than Gram-negative bacteria like *E. coli*, and *Salmonella typhimurium*.[8,9]

Chitosan has numerous advantages in food preservation, being produced from shrimp shells, which constitute a significant waste resource in Vietnam. Therefore, research on the production of chitosan films will contribute to improving the utilization of waste and diversifying the use of chitosan in the preservation of agricultural products.

Materials and Methods

Reagents

Acetic acid and sodium acetate trihydrate were obtained from Xilong Chemical Co. and Shanghai Chemical Co. (China), respectively.

Preparation of chitosan films

Chitosan with deacetylation degrees of 70%, 80%, and 90% were produced from shrimp shells collected from Nha Trang University, Vietnam, and stored in the laboratory at room temperature. The chitosan was diluted to a 1% concentration, soaked for 2 days, and stirred using a non-heating magnetic stirrer until fully dissolved. The stirring was done for approximately 2 h, and then allowed to stand for 24 h. The chitosan solution was then poured onto plastic dishes

measuring 7 cm x 15 cm (with a membrane thickness of 0.2 mm), left undisturbed for 30 min to set, and subsequently dried at 40°C for 6 h. After drying, the membrane was peeled off from the dish and stabilized at a temperature of 12°C.[10]

Determination of the Stability of Chitosan Films

The physical stability on storage of the 1% chitosan films with deacetylation degrees of 70%, 80%, and 90% was assessed by measuring the mechanical properties (tensile strength, elongation at break, and elastic modulus), and water vapour permeability of the film according to previously reported procedures.[11-16]

The thickness of the film was maintained at 0.02 mm (measured with a panme gauge with an accuracy of 0.02 µm), the temperature was maintained at 12°C, and the pH was maintained at 3.6 for film with deacetylation degree of 70%, and at pH 3.8 for films with deacetylation degrees of 80% and 90%. All the parameters were measured every 5 days for a period of 35 days.

Measurement of tensile strength, elongation at break, and elastic modulus

Prior to measurement, the films were conditioned to a stable environment with controlled temperature and humidity for 24 h. The measurements were carried out using an Instron structural testing device, where a sample of dimension 20 mm × 120 mm was subjected to an axial tensile force at a constant speed of 5 mm/s. The tensile force (F) and elongation (%) were recorded until the sample ruptured.

Measurement of water vapour permeability

The water vapour permeability (WVP) of the film was determined following the AFNOR standard method, NF H00-030 (1974), designed for thin film materials. The chitosan films were maintained at a relative humidity of 84% for 48 h using a saturated KCl solution. Subsequently, the films were securely clamped between two silicon cushion pads in a moisture measuring flask containing saturated potassium acetate solution (aw = 0.22). These flasks were placed in a sealed container containing a saturated potassium chloride solution (aw = 0.84) to create a moisture gradient on both sides of the membrane. Water vapour permeability was calculated based on the measurement of the increase in the mass of the flask.

Determination of the antifungal activity of chitosan films

The antifungal activity of chitosan films with deacetylation degrees of 70%, 80%, and 90% was assessed against *Colletotrichum gloeosporioides* using the disc diffusion method.

Colletotrichum gloeosporioides fungal strain was cultivated on potato dextrose agar (PDA) in a Petri dish incubated at 37°C for 4 days. After the fungal mycelium has reached a diameter of 30 mm, the chitosan films with a diameter of 6 mm each were placed on the fungal growth medium, and then the plates were incubated at 37°C. The antifungal activity of the chitosan films was measured based on the inhibition zone diameter around the chitosan films.[17] The inhibition zone diameter was measured every 5 days for 30 days.

Statistical analysis

The experiments were done in triplicates, and data were analyzed using the Statgraphics Centurion 16.1 program (Copyright (C) PP, USA) and Excel 2016 software. Differences between mean values were analysed using One-way analysis of variance (ANOVA), least significant difference (LSD), and Duncan's multiple range tests. P-value ≤ 0.05 was regarded as significant.

Results and Discussion

Effect of storage time on water vapour permeability of chitosan films

The adsorption equilibrium of chitosan film for water vapour was measured by the mass gain of the chitosan film within a controlled humidified environment. The humidity of the incoming air has been found to significantly affect chitosan film permeability.[18]

As shown in Figure 1, the average water vapour permeability through the chitosan film was found to be highest in the film with 80% degree of deacetylation, with a mean value of 4.775 x 10^{-11} $g.m^{-1}.s^{-1}.Pa^{-1}$. This was closely followed by the chitosan film with 90% degree of deacetylation which had an average water vapour permeability of 4.681 x 10^{-11} $g.m^{-1}.s^{-1}.Pa^{-1}$, while the chitosan film with 70% degree of deacetylation had the lowest water vapour permeability of 4.646 x 10^{-11} $g.m^{-1}.s^{-1}.Pa^{-1}$. The lower the permeability of the film, the more suitable it is for food preservation due to its ability to reduce moisture exchange. This agrees with the findings of Spangenperg *et al*. (2008).[19] The permeability of the film increased rapidly from the beginning until it reached a maximum value on the seventh day, after which it continued to increase abeit insignificantly. The permeability through the chitosan film is controlled by the supplied moisture. The films were placed in an environment with a specific relative humidity, causing the water vapour permeability process to be influenced by the difference in relative humidity between the outside (84%) and inside (22%) of the film.[20]

Effect of storage time on the mechanical properties of chitosan films

Following the production of chitosan films of three deacetylation degrees (70%, 80%, and 90%) with a film thickness of 0.02 mm, the films were maintained at 12°C before measurement of their mechanical properties (tensile strength, elongation at break, and elastic modulus) as a measure of their stability over time. The results of the assessment of the mechanical properties of the chitosan films are presented in Figures 2, 3, and 4 below.

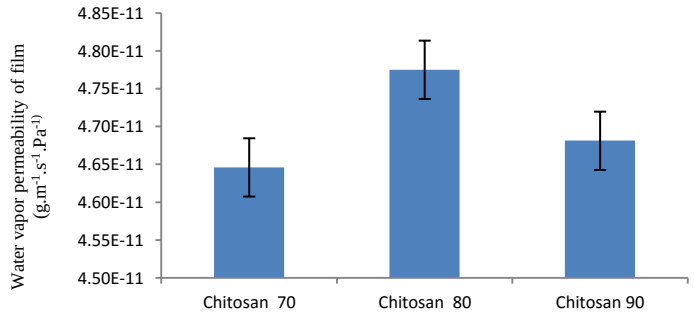

Figure 1: Water vapour permeability of chitosan films

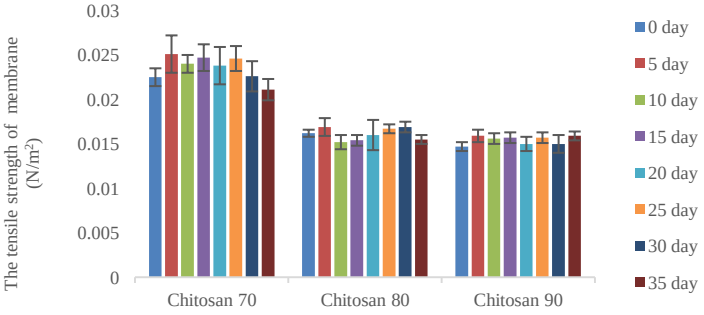

Figure 2: Tensile strength of chitosan films over 35 days period

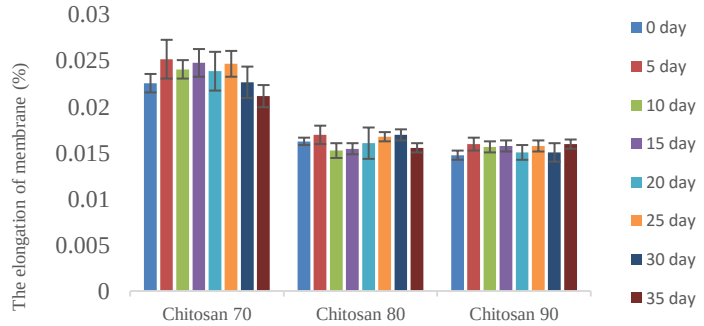

Figure 3: Elongation at break of chitosan films over 35 days period

For tensile strength, chitosan films with deacetylation degrees of 80% and 90% exhibited higher tensile strength than that with deacetylation degree of 70%. However, there was no significant difference ($P > 0.05$) over the observation period in the tensile strength of chitosan films at the different deacetylation degrees of 70%, 80%, and 90%.

With respect to elongation at break, chitosan films with deacetylation degrees of 80% and 90% demonstrated a significantly ($P < 0.05$) higher elongation at break than the film with a deacetylation degree of 70%. The elongation of chitosan films at deacetylation degrees of 70%, 80%, and 90% remained consistent over the observation period in contrast to the tensile strength, which increased as the degree of deacetylation increases, with a more substantial increase when approaching 100% deacetylation.[21]

For elasticity (elastic modulus), chitosan films with deacetylation degrees of 80% and 90% exhibited lower elasticity patterns compared to the film with 70% deacetylation. There was no significant difference in the elasticity of chitosan films at deacetylation degrees of 70%, 80%, and 90% over the observation period of 35 days. The elasticity pattern is used to assess the flexibility of the film, where a lower elasticity pattern indicates greater flexibility.

From the above observations, it is evident that there are differences between the deacetylation degrees, particularly with a clear distinction between chitosan films with a deacetylation degree of 70% and those with degrees of deacetylation of 80% and 90%. Overall, over the observation period, chitosan films at deacetylation degrees of 70%, 80%, and 90% were stable, which suggest that chitosan, being a natural polymer, functions as a cyclic complex, with excellent stability.

Antifungal activity of chitosan film against Colletotrichum gloeosporioides

The antifungal activity test was conducted using the disc diffusion method. The inhibition zone diameter on the agar medium was used as a measure of the antifungal efficacy of the chitosan films.

The antifungal activity of the chitosan films is presented in Figure 5 and Table 1. Chitosan films demonstrated high antifungal effectiveness by inhibiting the spore germination of *Colletotrichum gloeosporioides* (Figure 5). The antifungal activity of the chitosan films at three deacetylation degrees (70%, 80%, and 90%) varied in a time- and concentration-dependent manner. Chitosan films with a 90% degree of deacetylation exhibited the highest antifungal activity with the highest inhibition zone diameters over the stability period of the film. This was followed by the chitosan film with 80% degree of deacetylation, and lastly the chitosan film with 70% degree of deacetylation (Table 1). It is important to note that the antifungal activity of the chitosan films decreased significantly over time, showing the highest inhibition zone diameters on day 5, and the lowest inhibition zone diameters on day 30 for all the three types of films.

Conclusion

The findings from the present study have shown that chitosan films with deacetylation degrees of 70%, 80%, and 90%, and membrane thickness of 0.02 mm are stable over time at controlled environmental conditions. The tensile strength, elongation at break, elastic modulus, and water vapour permeability of the films did not undergo significant alterations after 35 days. The chitosan film at all three deacetylation degrees exhibited excellent antifungal activity against *Colletotrichum gloeosporioides* in a time- and concentration-dependent manner. This suggests that chitosan could be utilized as protective films for food preservation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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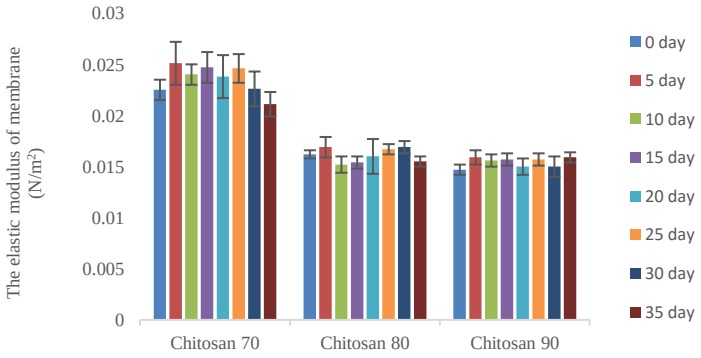

Figure 4: Elastic modulus of chitosan films over 35 days period

Table 1: Antifungal activity of chitosan films against *Colletotrichum gloeosporioides*

	Inhibition Zone Diameter (mm)		
Sample/Stability Time	**Chitosan film with 70% deacetylation degree**	**Chitosan film with 80% deacetylation degree**	**Chitosan film with 90% deacetylation degree**
Day 5	9.93 ± 0.69	11.15 ± 0.77	12.67 ± 1.03
Day 10	8.14 ± 0.43	9.30 ± 0.89	11.11 ± 0.81
Day 15	6.15 ± 0.18	7.06 ± 0.80	9.36 ± 0.45
Day 20	3.50 ± 0.71	4.84 ± 0.90	6.81 ± 0.85
Day 25	1.88 ± 0.41	2.56 ± 0.72	3.94 ± 0.48
Day 30	0.78 ± 0.73	1.12 ± 0.04	1.69 ± 0.47

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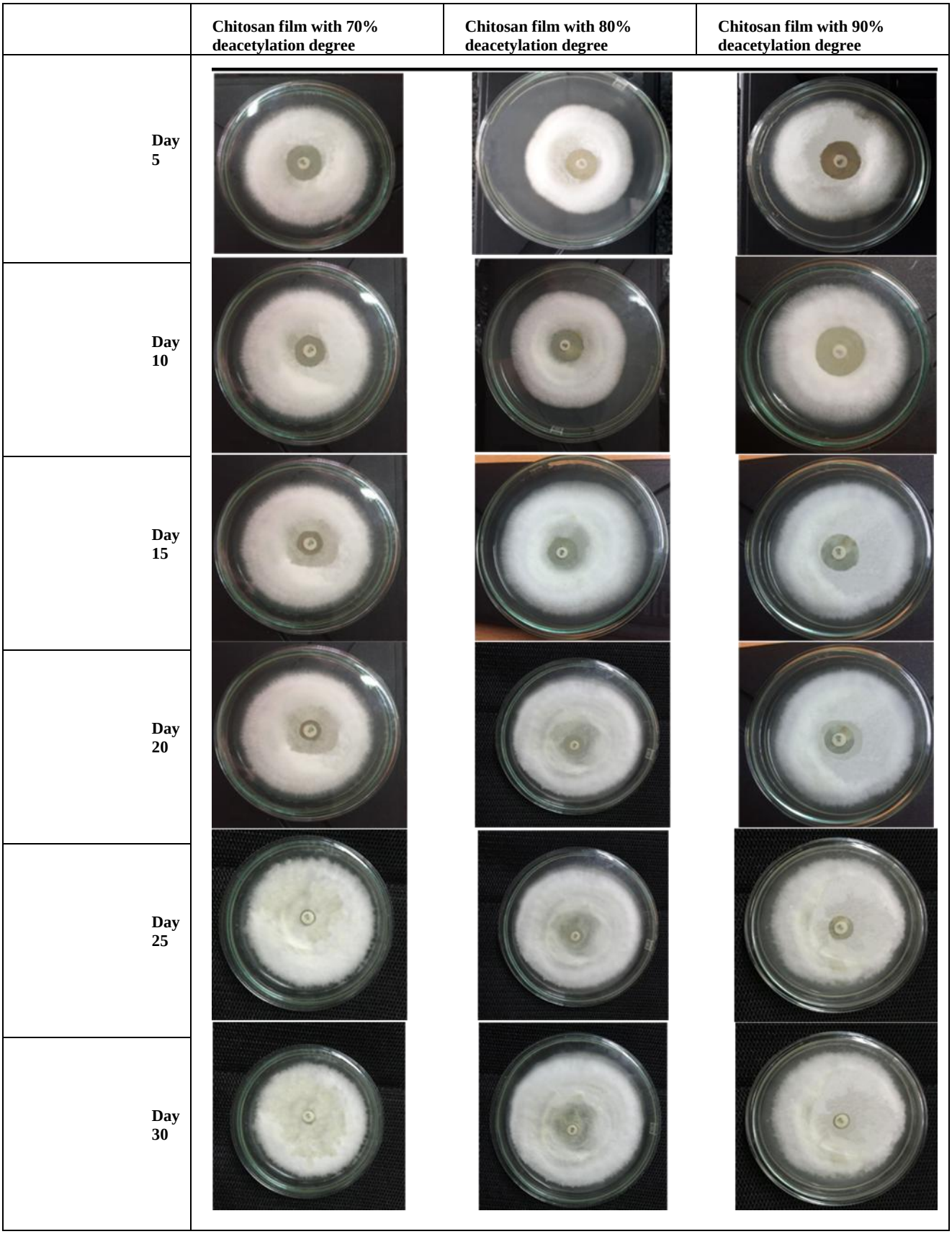

Figure 5: Petri dishes showing the antifungal inhibition zones of chitosan films against *Colletotrichum gloeosporioides*

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