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Preparation, Physicochemical Properties and *In Vitro* Antimicrobial Acitvities of Oligochitosan Against *Fusarium Moniliforme* and *Ralstonia Solanacearum*

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ABSTRACT

This study focuses on the synthesis and characterization of oligochitosan and its potential antimicrobial properties. Oligochitosan was produced by dissolving chitosan in 2% acetic acid and degrading it with 3% hydrogen peroxide at 60 °C for 4 h, resulting in a product with a molecular weight of 7.92 kDa. The antimicrobial effects were tested against the fungal pathogen *Fusarium moniliforme* and the bacterial pathogen *Ralstonia solanacearum* under *in vitro* conditions. The results indicated that oligochitosan demonstrated a concentration-dependent antifungal activity, achieving up to 60.17% inhibition of mycelial growth at 2000 ppm, which was higher than the inhibition observed with chitosan. However, neither chitosan nor oligochitosan exhibited antibacterial effects against *R. solanacearum*. The findings suggest that oligochitosan holds promise as an effective antifungal agent, though its antibacterial activity remains negligible. These results support the application of oligochitosan in agricultural and biological control, particularly for plant disease management.

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1. Introduction

Chitosan is a biopolymer with the chemical structure poly-2-amino-2-deoxy-β-D-glucose, which can be derived through the deacetylation process of chitin. This polymer has found widespread applications across various fields, including medicine, materials science, pharmaceuticals, food, and agriculture [1]. Among its derivatives, oligochitosan is produced by breaking down the chitosan chain using methods such as enzyme hydrolysis, irradiation, or the use of strong oxidizing agents. Recent interest has focused on oligochitosan with low molecular weight due to its solubility in water and its superior biological activity. These properties make oligochitosan highly applicable across diverse sectors, including agriculture. Several studies have demonstrated that oligochitosan holds considerable potential in inhibiting pathogen development, altering the morphology and structure of fungal cells, and disrupting their molecular organization, thereby stimulating plant defense responses [2].

Recent investigations have shown that oligochitosan can effectively inhibit the growth of plant pathogens both *in vitro* and in *vivo*. It does so by obstructing the radial growth of fungal pathogens such as *Fusarium solani*, *Puccinia arachidis*, *Alternaria alternata*, *Aspergillus niger*, and *Botrytis cinerea*. Furthermore, oligochitosan suppresses spore germination and reduces spore viability, as well as the growth of germ tubes, which in turn decreases the incidence of plant diseases and enhances the plant's defensive capabilities [2]. Consequently, oligochitosan presents a promising solution as a potential plant vaccine, contributing to the reduction of pesticide usage in agriculture. The objective of this study is to investigate the synthesis of low-molecular-weight oligochitosan and evaluate its effectiveness in combating several fungal and bacterial strains commonly responsible for plant diseases.

2. Materials and Methods

2.1. Materials and chemicals

Chitosan flakes (degree of deacetylation > 90%) derived from shrimp shells were obtained from S-Green Company (Ho Chi Minh City, Vietnam). Synthetic media PDA, LB, and TZCA were sourced

from Himedia (India). Fungal strains *Fusarium moniliforme* and *Ralstonia solanacearum* were isolated in previous studies and are stored in the microbiology laboratory at the Institute of Applied Technology, Ho Chi Minh City Branch.

2.2. Synthesis of oligochitosan

Oligochitosan was prepared via oxidative degradation of chitosan using hydrogen peroxide. The general synthesis procedure was as follows: Chitosan flakes (2.5 g) were completely dissolved in 100 mL of acetic acid solution with continuous stirring. The solution was then kept in a temperature-controlled water bath at 40 °C. Subsequently, a predetermined amount of hydrogen peroxide was added to initiate the degradation reaction. The mixture was maintained at a specific temperature and for a set duration. Upon completion, the reaction was stopped by neutralizing the excess acetic acid with a 10% NaOH solution until the pH reached 8–9, inducing the precipitation of the oligochitosan product. The resulting precipitate was recovered by centrifugation at 8,000 rpm for 10 minutes. The solid was then repeatedly washed with distilled water until the supernatant became neutral and finally dried at 50 °C to a constant weight.

To systematically evaluate the influence of key reaction parameters on the yield and properties of the synthesized oligochitosan, a one-factor-at-a-time method was employed. The investigation began by evaluating acetic acid concentrations of 1%, 2%, 3%, and 4% , from which 2% was selected as the suitable condition for subsequent experiments. Next, using the 2% acetic acid solution, the influence of H_2O_2 concentration was examined at levels of 1%, 2%, 3%, 4%, and 5% , with 3% H_2O_2 being identified as the most suitable concentration. Finally, under these settings, the effects of reaction temperature (40, 50, 60, and 70 °C) and duration (2, 3, 4, and 5 h) were evaluated.

2.3. Physicochemical characterization of oligochitosan

Viscosity of oligochitosan solutions was measured using an Otswald viscometer (Germany) in a 0.25M CH_3COOH and 0.25 M CH_3COONa buffer solution [3]. The intrinsic viscosity ([η]) was calculated using the formula:

$$[\eta] = \lim_{C \to 0} \left(\frac{\eta_r}{C} \right)^n \tag{1}$$

The molecular weight (Mw) of oligochitosan was determined using the Mark-Houwink equation:

$$[\eta] = K \times [M_w]^{\alpha} \tag{2}$$

where $K = 1.4 \times 10^{-4}$ and $\alpha = 0.83$ [4].

Solubility was assessed by dissolving 0.1 g of sample in 10 g of distilled water for 2 days, followed by filtration and drying the filtrate at 60 °C to a constant weight (m). Solubility (T%) was calculated as:

$$T(\%) = \frac{m}{D} \times 100\% \qquad \text{with D = 0.1 g} \tag{3}$$

The yield of oligochitosan was calculated as:

$$H = \frac{A}{B} \times 100\% \tag{4}$$

where A is the mass of oligochitosan after drying (g), and B is the initial chitosan mass (g).

The structural properties of oligochitosan were characterized using several methods: X-ray diffraction (XRD) was conducted on a Brucker D8 Advance instrument with a Cu anode (λ = 1.5406 Å). Fourier-transform infrared (FTIR) spectroscopy was performed on a JASCO FTIR/IR-4700 spectrometer, scanning between 4000 and 400 cm^{-1}. Thermal properties were evaluated using thermogravimetric analysis (TGA) on a Labsys TG/DSC-SETARAM analyzer. Samples (5 mg) were heated from room temperature to 600 °C at 20 °C/min in air.

2.4. In vitro antifungal activity against Fusarium moniliforme

Potato dextrose agar (PDA) medium was supplemented with oligochitosan/chitosan solutions at concentrations of 250, 500, 1000 and 2000 ppm. 7-day-old fungal discs (7 mm diameter) were inoculated at the center of the plates and incubated in dim light at room temperature. Colony diameter was measured after 3 and 7 days of incubation. The antifungal activity was calculated as:

$$H(\%) = \left(\frac{D-d}{D} \right) \times 100 \quad (5)$$

where D is the colony diameter on PDA without oligochitosan (control), and d is the colony diameter on PDA with oligochitosan.

2.5. Antibacterial activity against Ralstonia solanacearum

Antibacterial activity was evaluated using the agar disc diffusion method. 100 µL of bacterial suspension (10^8 CFU/mL) was spread on each PDA plate. Wells (7 mm in diameter) were punched in the agar and filled with 100 µL oligochitosan solution at concentrations of 500, 1000, 2000, and 4000 ppm, prepared in 0.1% and 1% acetic acid. The acetic acid solutions (0.1% and 1%) were used as controls. After 48 h of incubation at room temperature, the diameter of the inhibition zone was measured.

2.6. Statistical analysis

All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA and Tukey's test for statistical significance ($P < 0.05$), using Minitab 18.0 software.

3. Results and Discussion

3.1. Effects of reaction conditions on physicochemical and structural properties of synthesized oligochitosan

3.1.1. Acetic acid concentration

In this study, acetic acid was essential because chitosan is insoluble in neutral or basic water. However, excessively high acid concentrations can negatively impact the reaction between chitosan and hydrogen peroxide. While many acids like HCl, HNO_3, lactic acid or formic acid can also dissolve chitosan, acetic acid was chosen for its advantages. It is a weak acid, less corrosive, inexpensive, and safer for both humans and the environment. Therefore, we investigated the effect of acetic acid concentrations ranging from 1% to 4% on the yield, intrinsic viscosity, average molecular weight, and solubility of oligochitosan (Table 1). The data indicate that while acetic acid concentration does not affect the yield, it significantly influences the viscosity, molecular weight, and solubility of the resulting oligochitosan.

A slight decrease in viscosity and molecular weight was observed as the acetic acid concentration increased from 1% to 2%, but this difference was not statistically significant. However, when the concentration was raised to 4%, both viscosity and molecular weight increased significantly, with statistical differences compared to the other concentrations. Therefore, 2% acetic acid was selected as the suitable concentration for dissolving chitosan in subsequent oligochitosan synthesis experiments.

Table 1. *Effect of acetic acid concentration on the properties of oligochitosan. Reaction conditions: 1% H_2O_2, 40 °C for 1 h.*

CH_3COOH concentration (%)	Yield (%)	Viscosity (mPas)	Molecular weight (KDa)	Solubility (%)
1	91.22 ± 3.15^a	1.42 ± 0.04^b	66.97 ± 2.74^b	11.48 ± 1.28^a
2	**91.95 ± 3.16^a**	**1.38 ± 0.05^b**	**64.51 ± 2.87^b**	**12.79 ± 1.23^a**
3	92.71 ± 2.86^a	1.46 ± 0.05^b	69.25 ± 2.95^{ab}	12.61 ± 1.17^a
4	87.65 ± 1.80^a	1.59 ± 0.06^a	77.07 ± 3.43^a	12.43 ± 1.04^a

Means with different superscript letters in a columm are significantly different (P < 0.05).

The mechanism by which hydrogen peroxide oxidatively cleaves chitosan chains is as follows [5]. With a pKa of 11.6, Hydrogen peroxide is more acidic than water, hence it dissociates according to equation (6) to produce the perhydroxyl anion **(**HOO^-**).** This anion is unstable, which subsequently reacts with H_2O_2 to form the highly reactive hydroxyl radical (HO•) according to equation (7).

$$H_2O_2 \leftrightarrow H^+ + HOO^- \quad (6)$$

$$H_2O_2 + HOO^- \leftrightarrow HO\bullet + O_2^- + H_2O \quad (7)$$

Many studies show that hydroxyl radical reacts with carbohydrates rapidly, abstracting a C-bonded H atom according of the general equation (8):

$$RH + HO\bullet \leftrightarrow H_2O + R\bullet \quad (8)$$

The removal of a hydrogen atom from C-1, C-4, or C-5 of a sugar unit in polysaccharides would result in deamination and the breakage of the glycosidic bond, hence reducing the Mw of chitosan.

Based on the proposed mechanism, the oxidative scission of chitosan chains initiates with the dissociation of hydrogen peroxide, as shown in equation (6). Increasing the concentration of acetic acid leads to a higher concentration of H+ ions. This shifts the equilibrium of reaction (6) to the left, consequently reducing the formation of the perhydroxyl anion. As a result, the extent of oxidative chitosan chain scission decreases, yielding oligochitosan with a higher molecular weight. This increase in the molecular weight of oligochitosan was not statistically significant when acetic acid concentration was raised from 1% to 3%, but it became more pronounced at a 4% concentration (Table 1).

3.1.2. H_2O_2 concentration

The effect of H_2O_2 concentration on the properties of the obtained oligochitosan is summarized in Table 2. The results indicate that H_2O_2 concentration significantly influences the yield, viscosity, molecular weight, and solubility of the produced oligochitosan. Specifically, increasing the H_2O_2 concentration from 1% to 5% led to a decrease in yield, viscosity, molecular weight, and solubility of the oligochitosan.

Table 2. *Yield and properties of oligochitosan obtained at different H_2O_2 concentrations. Reaction conditions: 2% CH_3COOH, 40 °C for 1 h.*

H_2O_2 concentration (%)	Yield (%)	Viscosity (mPas)	Molecular weight (KDa)	Solubility (%)
1	93.44 ± 3.69^{a}	0.46 ± 0.02^{a}	17.04 ± 0.83^{a}	12.28 ± 1.08^{b}
2	88.72 ± 4.13^{a}	0.31 ± 0.02^{b}	10.56 ± 0.84^{b}	16.37 ± 1.01^{a}
3	$\mathbf{75.99 \pm 4.68^{b}}$	$\mathbf{0.25 \pm 0.01^{c}}$	$\mathbf{8.14 \pm 0.42^{c}}$	$\mathbf{17.05 \pm 1.28^{a}}$
4	67.81 ± 2.64^{bc}	0.23 ± 0.02^{c}	7.44 ± 0.50^{c}	17.34 ± 1.05^{a}
5	64.81 ± 1.79^{c}	0.22 ± 0.02^{c}	7.08 ± 0.50^{c}	17.82 ± 0.87^{a}

Means with different superscript letters in a columm are significantly different (P < 0.05).

At H_2O_2 concentrations of 1% and 2%, the yield of oligochitosan remained similar (93.44-88.72%). However, when the H_2O_2 concentration increased from 2% to 3%, a significant decline in yield, viscosity, and molecular weight was observed. Further increases in H_2O_2 concentration (from 3% to 5%) did not result in substantial changes in viscosity, molecular weight, or solubility. This trend is likely due to the increased concentration of oxidative OH^- radicals, which accelerate the hydrolysis of β-(1,4)-glycosidic bonds, thereby reducing viscosity, molecular weight, and increasing solubility [6]. Based on these findings, a 3% H_2O_2 concentration was determined to be acceptable for chitosan degradation.

Additionally, the color of the oligochitosan solutions darkened with higher H_2O_2 concentrations (Figure 1). This is likely due to the oxidation of chitosan into oligochitosan, which generates aldehyde groups from hydroxyl functional groups. These aldehydes then react with amino groups in oligochitosan

to form colored Schiff bases (imines).The formation of these imines with C=N double bonds, causes light absorption at different visible wavelengths. The increase in H_2O_2 concentrations resulted in higher imine concentrations and more intensed light absorption, making the product darker, turning from yellow to brown.

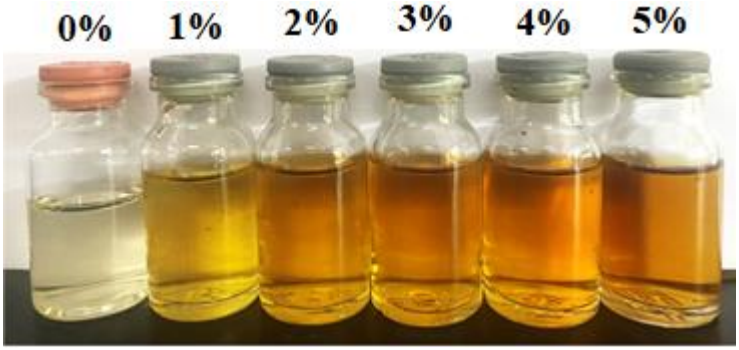

Figure 1. *Solutions of chitosan and oligochitosan samples produced with different H_2O_2 concentrations.*

3.1.3. Effects of temperature and reaction time

The effects of temperature and reaction time on the molecular weight and solubility of oligochitosan are presented in Table 3. The general trend observed in the Table is that increasing reaction temperature or time resulted in lower molecular weight, and higher solubility. This result makes sense because higher temperatures increase the speed and the extent of oxidation of chitosan and oligochitosan. While a longer reaction time does noy increase the reaction speed, it does give the newly formed oligochitosan more time in contact with hydrogen peroxide, making them more likely to be broken down into even shorter chains. As we explained in section 3.1.2 about the effect hydrogen peroxide concentration, increasing the extent of chitosan oxidation results in not only shorter oligochitosan molecules, but also more hydrophilic groups on those molecules. This, in turn, makes them more soluble.

Table 3 shows that at 60 °C with a reaction times of4 h, the molecular weight of the resulting oligochitosan decreased sharply from 21 kDa to approximately 6-7 kDa. Prolonging the reaction time or increasing the temperature beyond this point did not further reduce the molecular weight significantly, possible due to the depletion of hydrogen peroxide. These results are consistent with findings from [6]. Therefore, the temperature of 60 °C and reaction time of 4 h were selected for the chitosan depolymerization process.

Table 3. *Molecular weight and solubility of oligochitosan synthesized at different temperatures and reaction times. Reaction conditions: 2% CH_3COOH and 3% H_2O_2.*

Temperature (°C)	**Reaction time (h)**			
	2	**3**	**4**	**5**
	Molecular weight (kDa)			
40	26.62 ± 2.01^{a1}	24.02 ± 1.86^{a1}	16.14 ± 1.55^{a2}	14.36 ± 1.65^{a2}
50	25.99 ± 2.38^{a1}	21.37 ± 1.55^{a1}	14.93 ± 1.55^{a2}	13.50 ± 1.07^{a2}
60	11.94 ± 1.93^{b1}	9.54 ± 0.54^{b12}	$\mathbf{7.92 \pm 0.84^{b2}}$	7.67 ± 0.49^{b2}
70	11.47 ± 0.88^{b1}	8.43 ± 0.67^{b2}	4.78 ± 0.46^{b2}	6.73 ± 0.62^{b2}
	Solubility (%)			
40	12.39 ± 0.83^{b2}	14.34 ± 1.00^{b12}	15.81 ± 1.07^{b1}	16.78 ± 0.94^{b1}
50	12.91 ± 0.59^{b3}	14.84 ± 0.77^{b22}	15.97 ± 0.47^{b2}	17.36 ± 0.66^{b1}
60	16.11 ± 0.48^{a3}	16.37 ± 0.52^{ab23}	17.62 ± 0.68^{ab12}	18.53 ± 0.57^{ab1}
70	15.87 ± 0.66^{a2}	16.52 ± 0.44^{a2}	18.24 ± 0.56^{a1}	19.79 ± 0.73^{a1}

Different superscript letters in a column indicate significant differences (P<0.05). Different superscript numbers in a row indicate significant differences (P<0.05).

3.1.4. FTIR spectroscopy

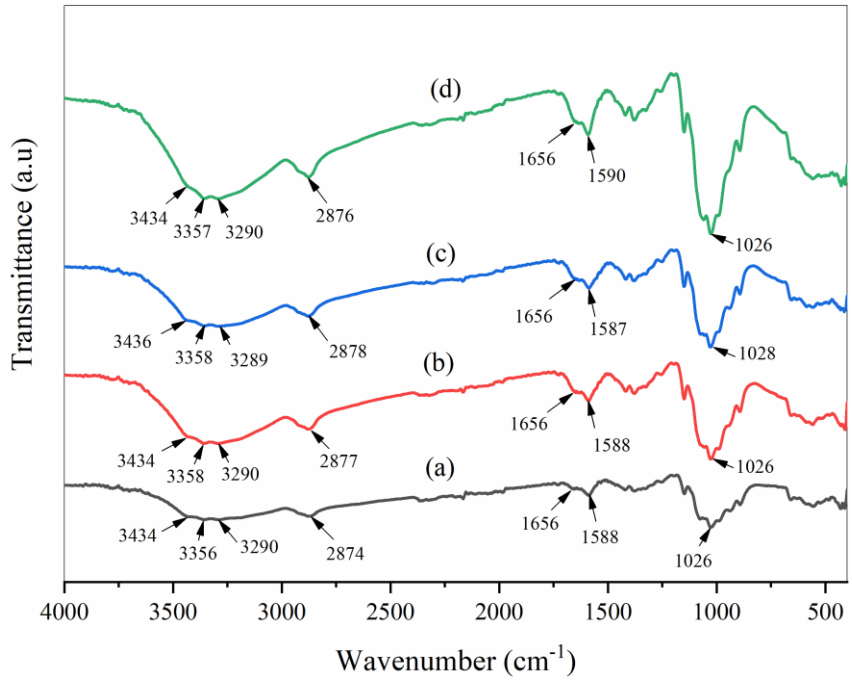

Figure 2. *FTIR spectra of chitosan (a) and oligochitosan samples (b,c,d) (b) 2% H_2O_2 at 70 °C; (c) 5% H_2O_2 at 70 °C; (d) 5% H_2O_2 at 40 °C*

FTIR spectroscopy (Figure 2) was employed to compare the chemical structures of chitosan and oligochitosan. Both materials display highly similar spectra. A significant broad absorption band is present around 3434-3356 cm^{-1}, attributed to overlapping O-H and primary N-H stretching, indicative of extensive hydrogen bonding; secondary N-H stretching is also noted near 3290 cm^{-1}. Characteristic polysaccharide C-H stretching vibrations occur near 2874–2878 cm^{-1}. Amide I (C=O stretch) and amide II (N-H bend) bands are clearly visible near 1656 cm^{-1} and 1587–1590 cm^{-1}, respectively. Vibrations corresponding to CH_2 bending appear around 1417–1421 cm^{-1}, while CH_3 deformation occurs near 1377 cm^{-1}. Furthermore, the asymmetric C-O-C stretch of the glycosidic backbone is found near 1148–1150 cm^{-1}, C-N stretching near 1323–1325 cm^{-1}, and C-O stretching vibrations around 1026–1028 cm^{-1}. Finally, the characteristic absorption peaks for β-(1,4)-glycosidic linkages are observed near 890–893 cm^{-1}. Overall, the FTIR spectral data confirm that oligochitosan largely preserves the fundamental functional group profile of the parent chitosan [7], [8].

3.1.5. XRD analysis

The crystallinity of oligochitosan and the original chitosan was analyzed by X-ray diffraction (XRD), as shown in Figure 3. Both chitosan (Figure 3a) and oligochitosan (Figures 3b, c) exhibited a broad diffraction peak at around 22.24°, 19.58°, and another peak near 10.26°, typical of crystalline regions in chitosan. However, the peak around 10° in oligochitosan was significantly lower than that of the chitosan sample, likely due to the disruption of its crystalline structure caused by the oxidation of H_2O_2. These results are consistent with findings by [9], [10] and support the hypothesis of amorphization due to H_2O_2 treatment [11].

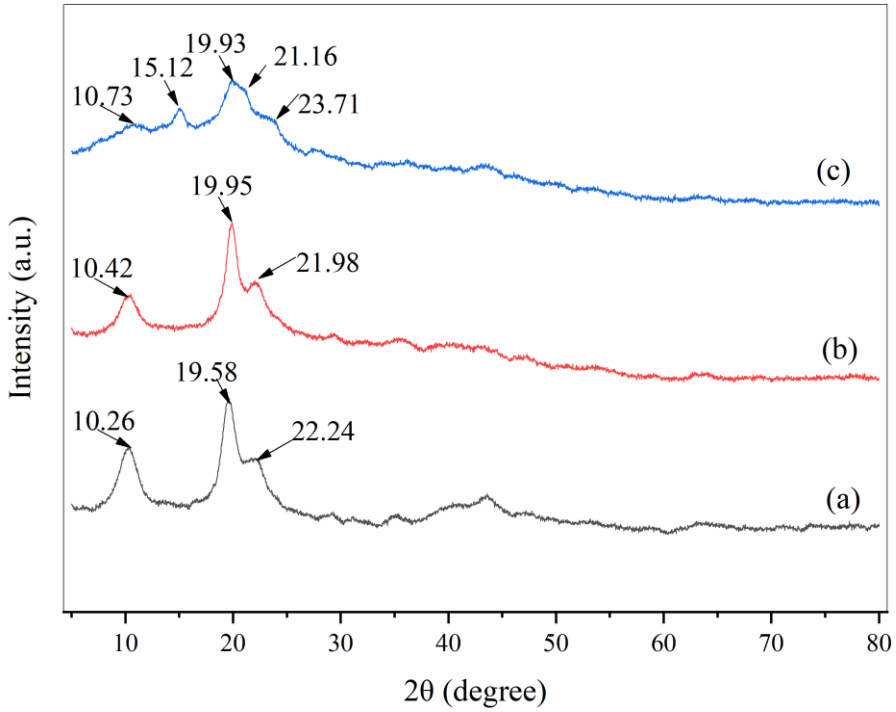

Figure 3. *XRD patterns of chitosan (a) and oligochitosan (b, c) (b) 2% H_2O_2 at 70 °C; (c) 3% H_2O_2 at 60 °C.*

3.1.6. Thermogravimetric analysis

The thermal degradation of oligochitosan and the original chitosan is shown in the thermogravimetric analysis (TGA) curve (Figure 4). The results indicate three distinct temperature ranges where significant weight loss occurs. Between 46°C and 110 °C, chitosan lost 7.07% of its weight, while oligochitosan lost 12.74%. This higher weight loss in oligochitosan may be due to the presence of volatile light compounds in both chitosan and oligochitosan samples.

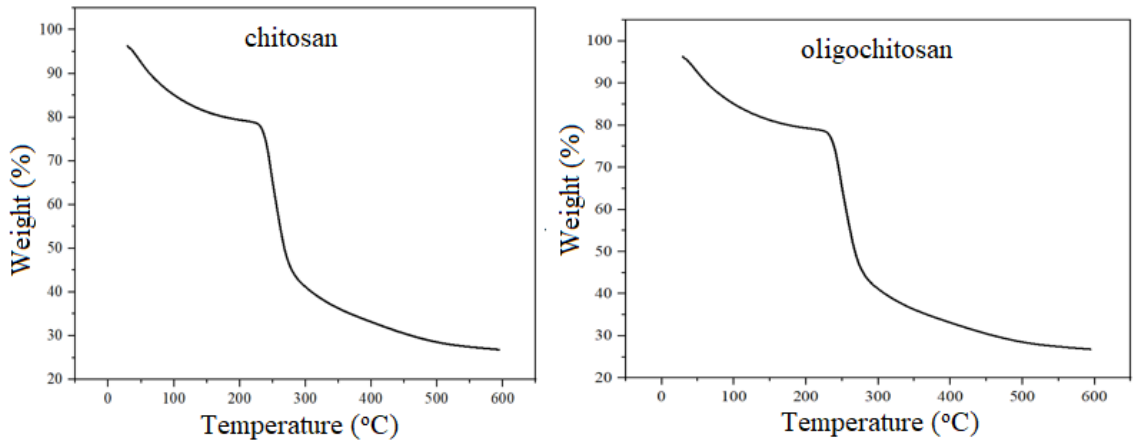

Figure 4. *TGA results of chitosan and oligochitosan prepared by treating with 3% H_2O_2 at 70 °C for 5 h*

From 263°C to 310 °C, chitosan lost 45.60% of its weight, whereas oligochitosan lost 41.22%, indicating that the main degradation occurred in this temperature range. Although the overall weight loss for both samples was similar, the onset of degradation for oligochitosan (263–301°C) occurred at a lower temperature compared to chitosan (269–310 °C), suggesting that oligochitosan has lower thermal stability than chitosan.

At temperatures above 400 °C, oligochitosan continued to lose an additional 6.77% of its weight, a phenomenon not observed in chitosan. This continued weight loss in oligochitosan is likely due to the breakdown of its polymeric structure. This difference in thermal behavior can be attributed to variations in structure and molecular weight between chitosan and oligochitosan. Furthermore, oligochitosan treated with H_2O_2 exhibited reduced thermal stability, which is likely due to the strong oxidizing effect of H_2O_2, leading to the breakdown of intramolecular interactions and partial molecular structure degradation [5].

3.2. Antifungal activity against Fusarium moniliforme

Table 4. *Inhibition of Fusarium moniliforme mycelium growth by chitosan/oligochitosan after 7 days*

Supplement concentration (ppm)	Mycelial growth inhibition (%) after 7 days	
	Chitosan	Oligochitosan
250	$2.23^{d} \pm 0.74$	$11.21^{d*} \pm 1.13$
500	$15.99^{c} \pm 3.74$	$20.35^{c*} \pm 1.18$
1000	$48.70^{b} \pm 1.49$	$54.28^{b*} \pm 1.14$
2000	$53.90^{a} \pm 1.21$	$60.17^{a*} \pm 1.13$

Different letters indicate significant differences (P<0.05). Asterisks (*) indicate statistical significance (P<0.05) between chitosan and oligochitosan at the same concentration.

The antifungal effects of oligochitosan and chitosan are summarized in Table 4 and Figure 5. Both oligochitosan and chitosan inhibited the growth of *F. moniliforme* mycelium, with inhibition increasing as the concentration of the samples increased. The antifungal activity remained consistent throughout the observation period. Oligochitosan exhibited a higher inhibitory effect than chitosan. Specifically, the inhibition by chitosan ranged from 2.23% to 53.90%, while oligochitosan inhibited mycelial growth from 11.21% to 60.17% at concentrations of 250 to 2000 ppm. These results demonstrate that both oligochitosan and chitosan can effectively inhibit the growth of *F. moniliforme*, consistent with previous studies [12], [13]. The higher efficacy of oligochitosan (with lower molecular weight) compared to chitosan aligns with findings for other fungi, such as *Botrytis cinera* [14], *Aspergillus niger* [15] and *Fusarium avenaceum* [16].

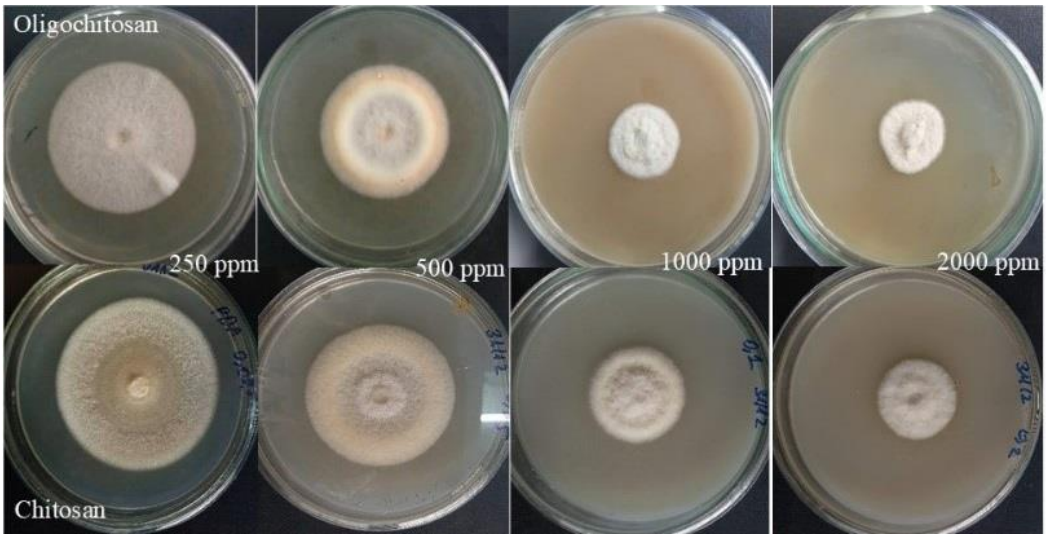

Figure 5. *Inhibition of Fusarium moniliforme mycelial growth by oligochitosan*

3.3. Antibacterial activity against Ralstonia solanacearum

Figure 6 illustrates the antibacterial activity of chitosan and oligochitosan against *R. solanacearum*. Neither chitosan nor oligochitosan exhibited any antibacterial effects when dissolved in 0.1% acetic acid

at concentrations ranging from 500 to 4000 ppm, as no inhibition zone was observed (Figure 6a, c). However, when dissolved in 1% acetic acid (Figure 6b, d), an inhibition zone appeared in all treatments, including the control (CH_3COOH 1%), though no significant difference was observed between the treatments. These results suggest that oligochitosan and chitosan, when dissolved in water or very dilute acetic acid do not inhibit *R. solanacearum*. In contrast, higher concentrations of acetic acid enhanced the inhibitory effect, likely due to the acetic acid itself, which could be responsible for the antibacterial activity [17].

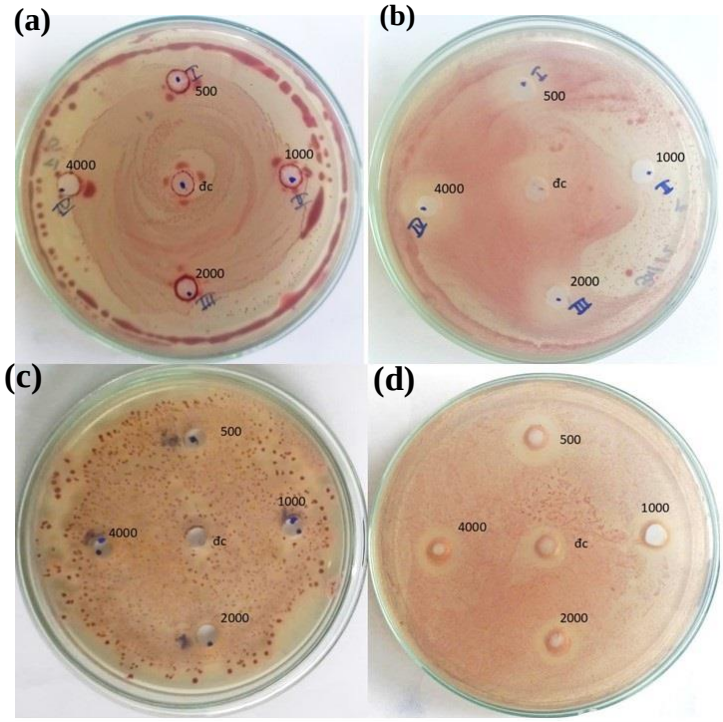

Figure 6. *Antagonistic images of chitosan and oligochitosan at 500, 1000, 2000, and 4000 ppm against Ralstonia solanacearum on agar plates after 48 h. (a, b): Chitosan dissolved in 0.1% and 1% CH_3COOH; (c, d): Oligochitosan dissolved in 0.1% and 1% CH_3COOH*

4. Conclusions

The study successfully synthesized oligochitosan by dissolving chitosan in 2% acetic acid and degrading it using 3% H_2O_2 at 60 °C for 4 h, yielding oligochitosan with a molecular weight of 7.92 kDa. The antifungal activity of oligochitosan was significantly higher than that of chitosan, effectively inhibiting the growth of *Fusarium moniliforme* at concentrations from 250 to 2000 ppm, with inhibition ranging from 11.21% to 60.17%. In contrast, neither chitosan nor oligochitosan showed antibacterial activity against *Ralstonia solanacearum*. These findings suggest that oligochitosan has promising potential as an antifungal agent but has limited antibacterial efficacy.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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