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# *IN VITRO* ANTIFUNGAL ACTIVITY OF CHITOSAN DERIVED FROM SHRIMP CO-PRODUCTS AGAINST PATHOGENIC FUNGI ISOLATED IN VIETNAM

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## ABSTRACT

The global overuse of antibiotics and agrochemicals in Vietnam leads to antibiotic resistance, health risks, and environmental damage. This study evaluates *in vitro* antifungal properties of different types of shrimp waste-derived chitosan against Vietnamese agricultural fungi as a sustainable alternative to chemical fungicides. Several pathogenic microbe strains were isolated and identified by morphological and molecular gene sequencing: *Neoscytalidium dimidiatum* causing brown spot on dragon fruit; *Fusarium fujikuroi* & *Fusarium subglutinans* causing banana crown rot; *Fusarium oxysporum* & *Fusarium odoratissimum* causing banana stem rot; *Lasiodiplodia theobroma*e causing fruit rot & *Colletotrichum queenslandicum* causing anthracnose on passion fruit; *Fusarium equiseti* & *Fusarium napiforme* causing swollen swim bladder on striped catfish. The antifungal properties of several chitosan types were investigated following the inhibition of the fungal mycelial growth method. CTIC15 & OLIC25 demonstrated significant fungal growth inhibition from 90% to 100% at 0.328 g/L to 0.625 g/L for all isolated fungal strains. Chitooligosaccharide COSL02 exhibited an antifungal effect against *L. theobromae*, *F. oxysporum*, *N. dimidiatum*, and *F. odoratissimum* with inhibition rates from 53.11 ± 2.74% to 100 ± 0.00% at 0.438 g/L to 0.876 g/L. Low molecular weight LV01 displayed broad-spectrum antifungal efficacy, excluding *F. subglutinans*, with inhibition rates from 74.11 ± 10.36% to 100 ± 0.00% at 0.2 g/L, and above 42.08 ± 5.87% at 0.1 g/L. Medium molecular weight MV01 shared comparable antifungal potency to LV01, except for *F. equiseti* and *N. dimidiatum*, with inhibition rates from above 74.09 ± 7.09% to 100 ± 0.00% at 0.2 g/L, and above 58.77 ± 0.87% at 0.1 g/L. This study suggested chitosan (shrimp waste) could serve as an effective and sustainable alternative to chemical fungicides in controlling pathogenic microbes.

**Keywords:** Antifungal activity, chitosan, disease control, postharvest preservation, shrimp waste

## INTRODUCTION

Antibiotics and agrochemicals encompass a wide range of chemical compounds utilized in agriculture to impede pathogen growth (Manyi-Loh *et al.*, 2018). The global agricultural industry, including horticulture, livestock husbandry and aquaculture, crucial for food security and economic sustainability, heavily relies on these substances. However, the escalating demand often leads to overuse, raising concerns about potential negative impacts. Carrique-Mas *et al.* (2020) reported that antibiotic use in animals in Vietnam is 1.6 times higher than in Europe, while horticulture sees massive pesticide usage of 52,000 tons, primarily 83% chemical-based, affecting product prices and global competitiveness. Despite Vietnamese government regulations, the misuse of antibiotics and agrochemicals, particularly in household farms, remains a significant challenge. Research in Vietnam indicated that 100% of surveyed farmers overused pesticides (Schreinemachers *et al.*, 2020) or 30.9% of surveyed farmers in a province of the central region failed to remember the last pesticides' name they used (Chau *et al.*, 2019) emphasizing the need for effective management and awareness. The shared mechanisms of action between chemicals used in animal farming and those for humans necessitate cautious usage due to potential interactions between animals and humans. Excessive and indiscriminate use of these drugs in agriculture raises concerns about antibiotic resistance strains, imbalance of the natural microbiome, potential health risks, and epidemic outbreaks. The misuse of these pathogen-controlled chemicals can be attributed to factors such as farmer's low awareness, pressure for farming efficiency, consumer demand for intensive farming, a wide variety of medications in the market without adequate instructions for farmers, and a lack of effective eco-friendly solutions (Manyi-Loh *et al.*, 2018). Global concerns about antibiotic and antifungal resistance are driving calls for sustainable and responsible agricultural practices. Promoting prudent antibiotic and antifungal use, enforcing strict regulations, and investing in alternative disease management techniques, including biological controls and genetic engineering, are gaining attention as strategies to address these challenges.

Chitosan, a natural linear polysaccharide, is composed of glucosamine and *N*-acetylglucosamine units linked by β-1,4-glycosidic bonds, is derived from the deacetylation of chitin found in the cuticle of crustaceans, fungi, and insects. Chitosan is biocompatible, biodegradable, non-toxic, and exhibits various biological qualities, including antibacterial, antifungal, coagulating, bio-adhesive, and bio-stimulatory. These properties have made it the finest candidate for wide application in agriculture (Bautista-Baños *et al.*, 2016; Sharp, 2013), medicine (Bouhenna *et al.*, 2015; Dash *et al.*, 2011), cosmetics (Rieger, 2009), wastewater treatment (No & Meyers, 2000), food packaging (Cazón & Vázquez, 2019), and food industry (Manigandan *et al.*, 2018). Numerous investigations have estimated the capability of chitosan in antimicrobial mechanisms derived either from the electrostatic interactions that changed the permeability of cell membranes, which thereby caused cell deaths, or from binding to DNA/RNA and inhibiting synthesis of mRNA and proteins (Al-Hetar *et al.*, 2011; Long *et al.*, 2014, 2018; Lopez-Moya *et al.*, 2019; Ren *et al.*, 2021; Xing *et al.*, 2015).

Despite numerous natural bioactive compounds being thoroughly researched, the characteristics of biodegradable, biocompatible compounds with antimicrobial capabilities from chitosan and its derivatives pose a gigantic potential for countless industries, namely horticulture, aquaculture, livestock, pharmaceutical, and feed production (Alaa *et al.*, 2020; Felipe *et al.*, 2019). Chitosan's mode of action was attributed to both direct and indirect perceptions. Firstly, the electrostatic interactions between the positive charge of protonated reactive amino groups on chitosan and the negatively charged microbial surface changed the membrane's permeability and led to cell death. Secondly, the chitosan–DNA/RNA interactions emerged from the chitosan's ability to pass through microbial cell walls, bind with DNA and inhibit the synthesis of mRNA, which would disrupt protein synthesis and thereby cause cell death. Thirdly, chitosan was implied as a chelating agent with some essential nutrients or metal ions, which crucially affect bacterial and fungal development. Moreover, chitosan is an elicitor, which stimulates the production of various pathogenesis-related proteins and defense-related enzymes in plants, helping plants fight against pathogen invasion (Alaa *et al.*, 2020; Al-Hetar *et al.*, 2011; Felipe *et al.*, 2019; Xing *et al.*, 2015). In addition to antifungal mechanisms, chitosan exhibits inhibitory effects by means of directly reducing mycelial growth, hyphal sporulation and germination (Al-Hetar *et al.*, 2011), manifested itself in mycelial swelling, excessive branching, abnormal shapes and molecular disorganization (Ren *et al.*, 2021) or indirectly eliciting the plant/animal immunity defense system through activating antioxidant enzymes, pathogenesis genes, etc. (Lopez-Moya *et al.*, 2019). Nevertheless, the insolubility of chitosan in neutral aqueous solutions and its high viscosity in acid environments pose challenges to its practical applications (Long *et al.*, 2014, 2018).

Recent research in Vietnam has explored the effective use of chitosan in controlling pathogenic microbes responsible for agricultural diseases and enhancing natural defense systems against infections. Studies of Long *et al.* (2014, 2015, 2018) concluded that water-soluble chitosan inhibited and effectively controlled anthracnose and green mold diseases in chili peppers, mangoes, and oranges. Similarly, Nga & Bac (2021) found that a chitosan coating formula maintained postharvest quality and reduced anthracnose spoilage on mangoes. Another experiment by Du *et al.* (2015) demonstrated that chitooligosaccharide increased chitinase activity, enhancing resistance against *N. dimidiatum*'s white spot disease on dragon fruits.

However, there have been insufficient studies of using commercial byproduct-derived chitosan instead of standardized chitosan or synthesizing dataset related to the effects of different types of chitosan (molecular weight and degree of deacetylation) on various isolated strains of pathogens adapted to soils and climates from Vietnam. Consequently, the objective in this research was to evaluate the *in vitro* potency of different modified types of shrimp waste-derived chitosan as antifungal agents in controlling or treating pathogenic microbes isolated from Vietnamese agriculture for the prospective commercial application of extending fruits' shelf life and animals' survival rate.

## MATERIALS AND METHODS

### Plant and animal sampling

The banana (*Musa acuminata* Cavendish), dragon fruits (*Selenicereus undatus*) and passion fruits (*Passiflora edulis*) were obtained from a local farm in Southern Provinces, Vietnam. Fruits were classified as defects of physical injury or disease infection showing typical symptoms of crown rot on banana fruit, brown spots on dragon fruit and anthracnose/fruit rot on passion fruit.

The striped catfish (*Pangasianodon hypophthalmus*) was screened from a local farming pond in the Mekong Delta, Vietnam. The striped catfish showed peculiar signs of defective swimming, anorexia, and swollen abdomen as the typical symptoms of swollen swim bladder.

These representative specimens were then washed with tap water, instantly stored in ice-cold conditions, and then sent to the laboratory for analysis (Long *et al.*, 2018).

### Isolation and identification of pathogenic microbes

Isolation of pathogenic fungi followed the standard phytopathological procedures (Burgess *et al.*, 2008). Infected tissues were aseptically cut into small pieces (approximately 2 x 2 mm) from the margin where healthy and disease tissues remain together. Tissue sections were sterilized by dipping in 70% ethyl alcohol for 5 seconds, then rinsing in sterile water and draining on sterile paper tissue. The cut pieces were placed in the middle of potato dextrose agar (PDA, Himedia, India) & water agar (WA) medium supplemented with gentamicin (500 µL/L, Fresenius Kabi, Germany) and incubated at room temperature (25-28°C) until fungal colonies formation. Subsequently, pure cultures were obtained by culturing from a single germinated spore and maintaining it on PDA medium for later use (Akter *et al.*, 2018; Long *et al.*, 2018; Mejdoub-Trabelsi *et al.*, 2020).

The identification of the pathogenic microbes was based on their morphological, cultural traits and the analysis of the 28S DNA gene sequence by means of similarity to the range of 98-100% in percent identity, the superlative total score and the query cover as the highest homologue with that of other strains blasted on GenBank NCBI (National Center for Biotechnology Information, U.S), which were carried out at a third-party testing laboratory.

### Chitosan preparation

Commercial chitosan was obtained from Vietnam Food JSC (VNF), which was produced from shrimp waste (shell and head) collected from shrimp processing producers for exports. Chitosan was derived from whiteleg shrimp (*Litopenaeus vannamei*) cultivated in intensive farms in the Mekong Delta. Chitosan samples CTIC15, OLIC25, and COS were commercial products. Chitosan samples LV01 and MV01, in powder form, were prepared in liquid form with a concentration of 1.0% (w/v) in 1.0% (v/v) acetic acid (Xilong Scientific, China), and subsequently stabilized at room temperature for 24 hours to ensure complete solubilization (degree of deacetylation $\geq$ 75%, ash content $\leq$ 2%, protein content $\leq$ 2%). Later, proportional dilutions (1:5 and 1:10) $^{(*)}$ of all tested samples were then prepared, followed by effective dosages based on data from a farm-site preliminary test and correlated to the

recommended dosage of chitosan's effectiveness analysis (Table 1). manufacturer, including the cost-

**Table 1.** Characteristics of chitosan samples used in the investigation

| Features | CTIC15 | LV01 | MV01 | OLIC25 | COSL02 |
|---|---|---|---|---|---|
| Type | Low molecular weight chitosan (LMWC) | LMWC | Medium molecular weight chitosan (MMWC) | Chito-oligosaccharide (COS) | COS |
| Chitosan concentration (g/L) | 1.64 | 1.0 | 1.0 | 3.25 | 4.38 |
| Proportional dilution (*) | 1:5<br>1:10 | 1:5<br>1:10 | 1:5<br>1:10 | 1:5<br>1:10 | 1:5<br>1:10 |
| Final concentration (g/L) | 0.328<br>0.164 | 0.2<br>0.1 | 0.2<br>0.1 | 0.65<br>0.325 | 0.876<br>0.438 |
| Degree of deacetyl (%) | NI | 89.22 | 91.83 | NI | 87.56 |
| Viscosity (1% in acetic acid 1%, 30°C, cPs) | NI | 115 | 413.5 | NI | 2.55 |
| Moisture (%) | NI | 10.12 | 8.16 | NI | NI |
| Protein (%) | NI | 0.175 | 0.24 | NI | NI |
| Ash (%) | NI | 0.4 | 0.51 | NI | NI |
| Turbidity (NTU) | 13 | 15 | 17.8 | NI | NI |
| pH | 3.79 | 7.82 | 8.85 | 2.84 | 3.04 |
| Soluble solids content (Brix, %) | 3.0 | NI | NI | 4.0 | 5.0 |
| Content (ppm) | 16,400 | 10,000 | 10,000 | 32,500 | 43,800 |
| Solubility in water | Completely dissolved | Insolubility | Insolubility | Insolubility | Completely dissolved |
| Ingredients | LMWC, orange and grapefruit essential oils | 100% from shrimp shell | 100% from shrimp shell | 100% from shrimp shell | COS, orange and grapefruit essential oils |

NI: not indicated.

### *In vitro* antifungal activity of chitosan against pathogenic fungi

The antifungal properties of chitosan were investigated following the inhibition of the fungal mycelial growth method (Akter *et al.*, 2018; Long *et al.*, 2018; Mejdoub-Trabelsi *et al.*, 2020; Ramos-Guerrero *et al.*, 2020). Chitosan solutions and PDA were sterilized separately, cooled at 50-55°C, then mixed to obtain chitosan: PDA concentration ratios of 1:5 and 1:10 and following dispersed aseptically in 85-mm diameter Petri dishes. Chitosan-free PDA plate was used as a control.

Plugs of 8-mm diameter taken from 7 days old pure cultures of the pathogen were distributed centrally to the dishes and then incubated at room temperature. Measurement was performed when the control plate reached full growth. Results in three replications were recorded individually, and averages were calculated.

The percentage of inhibition of mycelial growth was calculated by the formula: $I\% = [(C_2 - C_1) / C_2] \times 100$, whereby $C_2$ = pathogen colony diameter in control (chitosan-free PDA) plates and $C_1$ = pathogen colony diameter in chitosan-treated plates (Al-Hetar *et al.*, 2011).

### Experimental design and statistical data analysis

Experimental data were obtained with triplicates and repeated at least twice. Data recorded were analyzed statistically by one-way ANOVA using GraphPad Prism 9 and Multiple T-test (Tukey's test) with significance level $p < 0.05$.

## RESULTS AND DISCUSSION

### Pathogenic microbe isolation

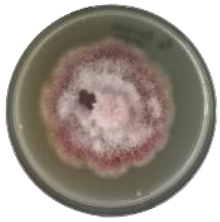
The sampled banana exhibited typical symptoms of crown rot; dragon fruits showed the signs of brown spot; passion fruits displayed typical symptoms of anthracnose/fruit rot, and; the striped catfish exhibited peculiar signs of defective swimming, anorexia, and swollen abdomen, characteristic of swollen swim bladder. Pathogenic microorganisms were identified based on their morphological features: mycelium color, colony pigmentation, colony diameters on PDA medium (Table 2). *Neoscytalidium dimidiatum* is characterized by numerous small, circular, reddish brown spots over fruits and stems. Soil-borne *Fusarium* species, which include four distinct categories: *Fusarium subglutinans*, *Fusarium fujikuroi*, *Fusarium oxysporum*, and *Fusarium odoratissimum*, are associated with symptoms such as wilted, yellowed leaves, vasculature browning, corm necrosis, and plant death. In passion fruit, *Colletotrichum queenslandicum* caused symptoms that include black acervuli, spherical to elongate, sparse, and black setae. *Lasiodiplodia theobromae* is responsible for dieback, stem-end rot, and fruit rot diseases. *Fusarium napiforme* and *Fusarium equiseti* infections in striped catfish manifest as a curved back, distended belly, difficulty with buoyancy, loss of appetite or difficulty feeding, lethargy, swollen swim bladders, and high mortality rates.

Dragon fruit, banana, passion fruit, striped catfish, and frogs are the primary Vietnamese food exports with significant economic value due to their high production volume and competitive advantage. Hence, large-scale cultivation and farming practices are necessary to maintain production. However, natural conditions have changed

and resulted in diverse pathogens that spread quickly, leading to colossal economic losses. This study identified *N. dimidiatum* as the causal agent of brown spot disease in dragon fruit, which was first reported by Lan *et al.* (2012) in the Chinese Mainland and expeditiously spread to dragon fruit-growing countries located in Southeast Asia (Du *et al.*, 2015). Acting as soil-borne saprophytes, persisted for several years in the form of chlamydospores (Abou-zeid *et al.*, 2020; Guarnaccia *et al.*, 2019), Fusarium wilt of banana, a highly destructive infection globally, is caused by coinfections of soil-borne *Fusarium* spp. (Al-Hetar *et al.*, 2011; Cannon *et al.*, 2022). Aside from causing banana Panama disease caused by *F. oxysporum* (Cannon *et al.*, 2022), the *Fusarium* pathogens in this research have also been reported in diverse host species, such as potato dry rot (Mejdoub-Trabelsi *et al.*, 2020; Ren *et al.*, 2021), tomato wilt (Alvarez-Carvajal *et al.*, 2020), or rice bakanae disease (Kim *et al.*, 2016). Anthracnose, another economically significant disease, is caused by *Colletotrichum* spp., affecting numerous woody and herbaceous crops during both pre- and postharvest periods as consequent symptoms of stem rot, dieback, and seedling blight (De Silva *et al.*, 2017; Long *et al.*, 2018; Muñoz *et al.*, 2009). This isolation of *C. queenslandicum* in passion fruit is consistent with its occurrence in other crops, such as chili (Akter *et al.*, 2018; De Silva *et al.*, 2017; Long *et al.*, 2018), tomatoes and grapes (Muñoz *et al.*, 2009), mango (Long *et al.*, 2015), red dragon fruit (Zahid *et al.*, 2015), or soursop (Ramos-Guerrero *et al.*, 2020). The presence of fungus *L. theobromae* in passion fruit as postharvest pathogens has been documented across a diverse range of agricultural crops, which were previously reported by Apai *et al.* (2008) as isolated from longan or Cannon *et al.* (2022) as isolated from grapevines. In aquaculture, a prevalent indication of fungal infection is a swollen swim bladder, which pinpointed *F. napiforme* and *F. equiseti* (Duc *et al.*, 2015; Pham *et al.*, 2015) as responsible for these infections that were similar to this isolation.

**Table 2.** List of pathogenic microbes isolated from infected agricultural samples

| Microbe strains | Diseased sample | Morphological characteristics | Identities (%) | Accession number | Illustrative colony image |
|---|---|---|---|---|---|
| *Neoscytalidium dimidiatum* | Dragon fruit | Fungal colony grew rapidly with cottony, the pigmentation was initially white and fluffy, then changed gradually to gray-green and darkened black | 99.61 | MW391102 |  |

| | | | | | |
|---|---|---|---|---|---|
| *Fusarium subglutinans* | Banana | Colony was cream-white mycelium with a peach mid-point in color and flat without cottony aerial mycelium in shape | 99.33 | MG274316 |  |
| *Fusarium fujikuroi* | Banana | Colony was white and smooth aerial mycelium with pink or vinaceous to violet undersurface on growth media and the thick-walled shape was capable of adhesion | 100 | CP023090 |  |
| *Fusarium oxysporum* | Banana | Colony changed from white to purple with a dark-purple center in color and oval to ellipsoid in shape | 100 | CP052041 |  |
| *Fusarium odoratissimum* | Banana | | 100 | LT571434 |  |
| *Lasiodiplodia theobromae* | Passion fruit | Colony changes from white to gray or dark green and sparse growth of aerial mycelium | 100 | OK056572 |  |

| | | | | | |
|---|---|---|---|---|---|
| *Colletotrichum queenslandicum* | Passion fruit | | 99.84 | MK298316 |  |
| *Fusarium napiforme* | Striped catfish | Colony grew fast, the color on the right and back sides was light white to pale pinkish, creamy, or yellowish and aerial mycelium grew abundantly on the culture medium | 100 | MH862670 |  |
| *Fusarium equiseti* | Striped catfish | | 100 | KC311517 |  |

**Effect of chitosan on mycelial growth of fungi**

Various forms of chitosan demonstrated distinct antifungal efficiency on different strains of fungi. The reduction of colony growth diameter of pathogenic fungi correlated to an increase of chitosan concentration (Figure 1). Chitosan's antifungal capabilities exhibited a progressive decrease followed the dilution rose from 1:5 to 1:10, indicating a decrease in chitosan concentration. Nonetheless, both CTIC15 and OLIC25 retained their effectiveness even when diluted.

The effectiveness of each type of chitosan against different pathogenic fungi was clarified. The terrific results were recorded by using the CTIC15 and OLIC25 chitosan against most pathogenic fungi at concentrations of 0.328 g/L and 0.650 g/L, namely *F. oxysporum*, *F. napiforme*, *F. odoratissimum*, *L. theobromae*, *C. queenslandicum*, *N. dimidiatum* , *F. fujikuroi*, and *F. equiseti* by 100%, 93.81%, 100%, 100%, 98.67%, 94.26%, 79.86%, 91.08% and 100%, 87.62%, 99.52%, 100%, 100%, 90.58%, 77.35%, and 97.81% respectively, with significant differences to the control. At a 1:10 dilution level of CTIC15 and OLIC25 with respective concentrations of 0.164 g/L and 0.468 g/L, antifungal activities exhibited slight changes at 86.55%, 87.68%, 90.54%, 86.99%, 94.21%, 92.41%, 68.54%, 86.11% and 74.76%, 77.75%, 81.04%, 100%, 80.43%, 81.97%, 66.71%, and 77.46% towards *F. oxysporum*, *F. napiforme*, *F. odoratissimum*, *L. theobromae*, *C. queenslandicum*, *N. dimidiatum, F. fujikuroi*, and *F. equiseti*. COSL02 displayed broad-spectrum antifungal properties, proportionally inhibiting the radial growth of *L. theobromae* by 100% / 98.37%, *F. oxysporum* by 96.41% / 65.98%, *N. dimidiatum* by 94.11% / 68.01%, *F.*

*odoratissimum* by 100% / 53.11% at 0.876 g/L / 0.438 g/L. However, the antifungal properties of COSL02 were only exhibited significantly at a 1:5 dilution ratio (equivalent to 0.876 g/L) in the treatment against *F. napiforme*, *C. queenslandicum*, *F. fujikuroi*, and *F. equiseti*, with inhibition rates of 63.39, 78.42, 100, and 89.31%, respectively, and the antifungal efficiency substantially dropped below 60% when diluted at 1:10 ratio (equivalent to 0.438 g/L) by 49.89, 37.06, 31.86, and 59.43%, respectively. Chitosan LV01 also showed wide-ranging antifungal potency on *L. theobromae*, *F. odoratissimum*, *F. oxysporum, F. equiseti, F. napiforme, C. queenslandicum*, *F. fujikuroi*, and *N. dimidiatum*, at both dilution levels by 100, 98.51, 97.95, 89.53, 89.23, 76.65, 74.88, and 74.11%, respectively, at 0.2 g/L and by 91.26, 84.18, 78.31, 78.90, 66.63, 42.08, 48.60, and 35.67%, respectively, at 0.1 g/L. Equivalently, chitosan MV01 demonstrated a comparable antifungal strength to LV01 against targeted pathogenic fungi, with exceptions for treatments on *F. equiseti* and *N. dimidiatum*. The respective inhibitory percentages at a chitosan concentration of 0.2 g/L were 92.07, 95.72, 97.95, 82.49, 75.56, and 74.09%, following the same order as LV01, while inhibitory percentages at a chitosan concentration of 0.1 g/L were 82.93, 81.00, 71.05, 77.11, 58.77, and 61.25%.

The potency of chitosan treatment on each pathogenic fungal strain was clarified. Treatment of *N. dimidiatum* demonstrated the effectiveness of CTIC15 (at both 0.328 g/L and 0.164 g/L) and OLIC25 (only 0.65 g/L) with inhibition rates of 94.26% / 90.58%, and 92.41%, respectively. For *F. subglutinans*, CTIC15, OLIC25, COSL02, and LV01 at concentrations of 0.328, 0.65, 0.876, and 0.2 g/L showed inhibition rates of 99.45, 92.04, 96.08, and 91.51%, respectively. Only COSL02 at a dosage of 0.876 g/L demonstrated 100% antifungal properties against *F. fujikuroi*. All chitosan samples exhibited antifungal properties above 90% against *F. oxysporum*, with CTIC15, OLIC25, COSL02, and LV01 at concentrations of 0.328, 0.65, 0.876, and 0.2 g/L achieving inhibition rates of 100, 100, 96.41, 97.95, and 97.95%, respectively. Similar efficacy was observed in the treatment against *F. odoratissimum*, with inhibition rates of 100, 99.52, 100, 98.51, and 95.72% for CTIC15, OLIC25, COSL02, and LV01 at concentrations of 0.328, 0.65, 0.876, and 0.2 g/L and for CTIC15 at a concentration of 0.164 g/L, respectively. For the treatment of *L. theobromae*, all chitosan samples involving CTIC15, OLIC25, COSL02, LV01, and MV01 at a 1:5 dilution ratio (equivalent to 0.328, 0.65, 0.876, 0.2, and 0.2 g/L) exhibited significant antifungal properties with inhibition rates of 100, 100, 100, 100, and 92.07%, and only chitosan samples involving OLIC25, COSL02, and LV01 at a 1:10 dilution ratio (equivalent to 0.325, 0.438, and 0.1 g/L) exhibited promising antifungal activities with rates of 100, 98.37, and 91.26%, respectively. In the treatment of *C. queenslandicum*, this fungus was found to be sensitive to CTIC15 and OLIC25 at concentrations of 0.328 and 0.65 g/L by 98.67 and 100%, respectively, and to CTIC15 at 0.164 g/L with a rate of 94.21%. *F. napiforme* was sensitive to CTIC15 at 0.328 g/L with a rate of 93.81%. Lastly, CTIC15 and OLIC25 at concentrations of 0.328 and 0.65 g/L showed substantial antifungal efficiency against *F. equiseti*, with inhibition rates of 91.08 and 97.81%, respectively.

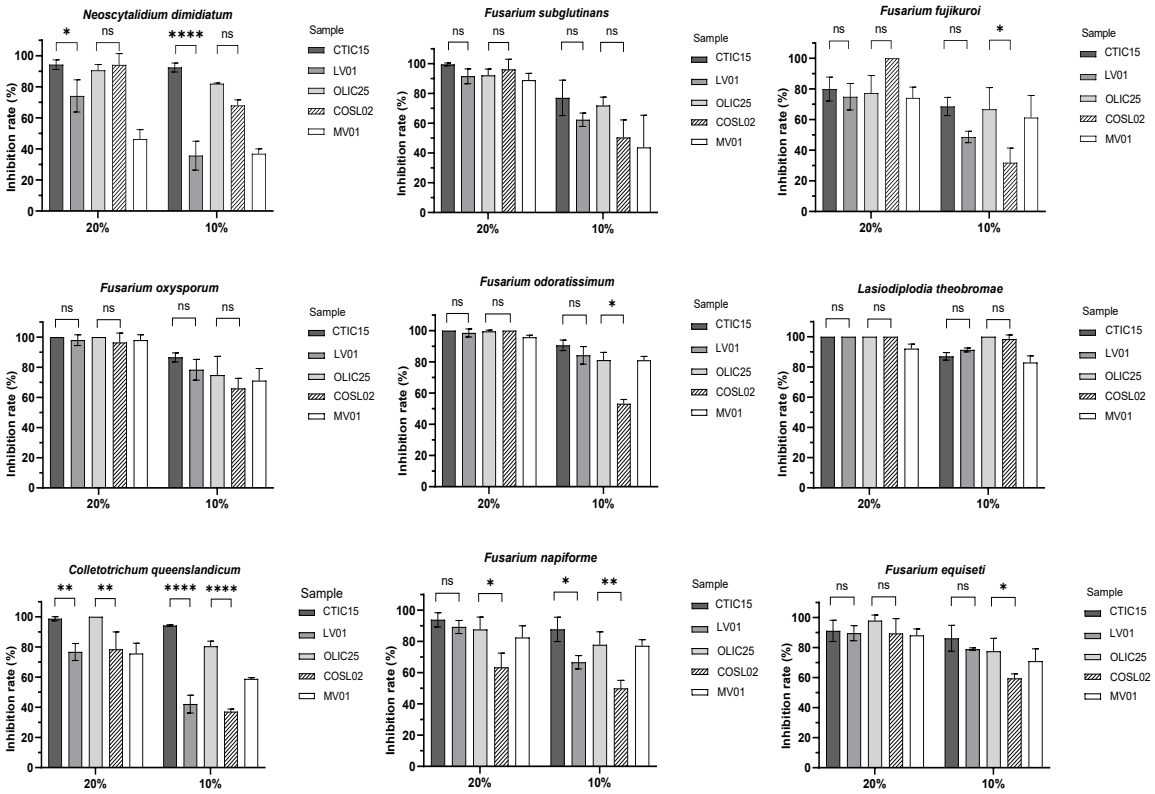



**Figure 1.** The effects of various chitosans (CTIC15, LV01, OLIC25, COSL02, MV01) on mycelial growth of several pathogenic fungi isolated from horticulture and aquaculture compared to control plates (chitosan-free PDA). The standard deviations are represented by the error bars, asterisk symbol (*) indicate the level of statistical difference at the significance level of 95% and letter (ns) indicate results do not differ statistically at the significance level of 5% (Turkey's test). Values display the arithmetic means of triplicates (n = 3). $p < 0.001$.

In summary, this investigation highlighted the ability of chitosan samples to inhibit 90% of microbial growth and cause cell death at suitable dosages. Not only do different types of chitosan affect the same fungal strains differently, but also the same type of chitosan affects different fungal strains differently. Results showed that treatment with LMWC LV01 and COS, either alone or incorporated with natural compounds, exhibited higher antifungal activities compared to treatments with MMWC MV01 across all tested fungal strains. These findings aligned with Hua *et al.* (2019), who revealed LMWC had a greater inhibitory effect than higher molecular weight chitosan in inhibiting spore germination and mycelial growth of *Botrytis cinerea* on kiwifruit. Additionally, the incidence of gray mold disease was reduced by 34% and 32% in the LMWC and HMWC treatments, respectively, three days post-inoculation. Another investigation by Hernandez-Lauzardo *et al.* (2008) pinpointed that high molecular weight chitosan had the lowest inhibitory effect on three isolates of *Rhizopus stolonifera* tested, which even more significantly affected spore development (sporulation, shape, and germination). The action mechanism of

LMWC could be explained via the study of Wang *et al.* (2017), which involves inhibiting ergosterol synthesis in fungal cell membranes, significantly reducing mitochondrial membrane potential (an early step in the apoptotic process), and allowing for easier penetration into the fungal cell due to its low molecular weight. This present study also highlighted the dependence of fungal development stage on fungal growth restriction by chitosan. The results indicated that *N. dimidiatum*, *C. queenslandicum*, *F. equiseti*, and *F. napiform*e, which reached full growth within 4-6 days, were less sensitive to chitosan compared to other fungal strains that took 7-10 days to reach full growth. This suggests that chitosan's inhibitory effect was lower on the faster-growing strains. Results were in agreement with study of Meng *et al.* (2010), which discovered both chitosan and oligochitosan displayed higher effectiveness on two fungi, *Alternaria kikuchinana* and *Physalospora piricola*, at the mycelial growth stage than that at the spore germination stage, most likely due to stronger drug resistance of fungal spores. Additionally, research by Palma-Guerrero *et al.* (2009) demonstrated that different cell types (conidia, germ tubes, and vegetative hyphae) show differential sensitivity to chitosan. Chitosan at a concentration of 0.1 mg/mL (with a molecular weight of 70 kDa, and a deacetylation degree of 79.6%) eliminated 99.15% of conidia after four minutes, with most conidia dying between 1.5 and 4 minutes. Conidial germlings died between 35 and 45 minutes, while vegetative hyphae started to die at 20 minutes and were all dead by 40 minutes.

Numerous investigations have demonstrated the significant multiple mechanisms of chitosan, namely antimicrobial, film-forming, host defense system's stimulation and constructive characteristics such as non-toxic, biodegradable, and biocompatible (Felipe *et al.*, 2019; Ramos-Guerrero *et al.*, 2020). Due to chitosan previous properties and the pressing need for alternative solutions in regulating agricultural diseases, this *in vitro* study illustrated chitosan antimicrobial activity, which varied based on microorganism strains, molecular weight, degree of deacetylation, concentration, and chitosan types. Likewise, the *in vitro* findings indicated that the inspected synergistic combinations of chitosan and essential oil, in the form of CTIC15 or OLIC25 formulations, demonstrated enhanced inhibitory effects on mycelial growth and colony morphology of *F. oxysporum*, *F. napiforme*, *F. odoratissimum*, *L. theobromae*, *C. queenslandicum*, and *N. dimidiatum* obtained at 0.328 g/L and 0.650 g/L chitosan concentration. Low molecular weight chitosan and chitooligosaccharide were fundamental substances of CTIC15 and OLIC25 that exhibited analogous antifungal spectrum and fungal strains comparable to CTIC15 and OLIC25. Nevertheless, the amalgamation of essential oil into chitosan not only enhanced but also sustained a greater antifungal potency over time when contrasted with the application of either essential oil or chitosan alone. The findings aligned with earlier research conducted by Oliveira *et al.* (2018), which indicated that the combination of chitosan and essential oil significantly hindered the growth of *C. asianum*, *C. fructicola*, *C. tropicale*, *C. siamense*, and *C. karstii*. Similarly, Sheikh *et al.* (2021) determined that the concurrent treatment involving 2% chitosan and MIC of essential oil resulted in the disruption of the fungal chitin cell wall and change membrane permeability. Besides, toxicity induced by chitosan against targeted fungi was likewise

contingent on the concentration of chitosan that the higher the concentration, the more potent the antifungal activity.

Furthermore, this study uncovered the impact of chitosan's molecular weight on its antifungal efficiency, demonstrating LMWC corresponds to enhanced antifungal activity. Experimental results showed that COS COSL02 exhibited the highest antifungal activity which could thoroughly inhibit the growth of *L. theobromae*, *N. dimidiatum*, and *F. odoratissimum* by 100, 94.11, and 100%, followed by LMWC LV01 with 100, 74.11, and 98.51%, while the least effective results compared to COSL02 and LV01 were observed at MMWC MV01 by 92.07, 46.31, and 95.72%. The similar results were also recorded in studies conducted by Li *et al.* (2008), which indicated that 50 kDa chitosan displayed the highest antifungal activity, while 140 kDa to 200 kDa chitosan were able to completely inhibit the growth of *Aspergillus niger* but numerous colonies emerged afterwards, and 800 kDa to 1000 kDa did not possess the antifungal properties but instead promoted the growth of *A. niger*. Additional findings conducted by Younes *et al.* (2014) suggested that the antifungal efficacy of chitosan was impacted by its molecular weight concerning *F. oxysporum*, however, there was no observed dependence on molecular weight with *A. niger*, which contrasted with the outcomes of our present investigation.

The pH value was another factor contributing to the variability in the antifungal effectiveness of chitosan, where higher activity was recorded at lower pH values. Results showed that 1% (v/v) acetic acid with a pH around 2.8 demonstrated greater antifungal potency compared to 1% (w/v) LMWC LV01 or MMWC MV01 prepared in 1% (v/v) acetic acid with a pH range of approximately 3.4-3.6, suggesting that the introduction of $H^+$ (acid solvent) to $NH_2$ groups (chitosan) contributed to raising the pH value of the resulting chitosan solutions. As per the findings of Ali *et al.* (2017) and Mustafa *et al.* (2023), various fungi were found to thrive within the pH range of 3.0-8.5, it was observed that a lower acidic pH value or higher alkaline pH value could impede the growth of fungi. Nevertheless, our findings indicated that when 1% (v/v) acetic acid was diluted by a factor of 10, its antifungal effectiveness diminished sharply. In contrast, when chitosan solutions underwent the same dilution, the efficacies either remained unchanged or slightly decreased. The results aligned with the discoveries of Younes *et al.* (2014), who highlighted the effects of pH on the antimicrobial activity of chitosan, with a greater activity at lower pH values where the protonation degree was larger. Similarly, Li *et al.* (2008) reported that antifungal activities of chitosan intensified as the pH decreased, where pH values of 3.0 or below displayed complete inhibition.

These results were in agreement with prior studies where water-soluble chitosan (85-90% deacetylation) at a concentration of 0.8% completely inhibited the mycelial growth of *Colletotrichum gloeosporioides* (Long *et al.*, 2015). Another investigation was conducted by Long *et al.* (2018) indicated that the potency of water-soluble chitosan (90 ± 5% degree of deacetylation) at 0.8% utterly prevented the development of *Colletotrichum capsici*. Mejdoub-Trabelsi *et al.* (2020) showed that chitosan (molecular weight 150 kDa, 75-78% deacetylated) applying *in vitro* at 4 g/L significantly decreased the mycelial growth of *F. oxysporum* by 88.4%. In addition, the maximum inhibition to sporulation of *F.*

*oxysporum* achieved 96.53% and increased gradually with increasing chitosan concentration (shrimp shell chitosan with 88% deacetylated, viscosity 370 cP at 0.5% in 0.5% acetic acid) (Al-Hetar *et al.*, 2011). Conjointly, Kim *et al.* (2016) reported the potent inhibitory effects of chitooligosaccharide or water-soluble chitosan (produced from high molecular weight 1900 kDa and 98.5% degree of deacetylation) on spore germination of all tested strains of *F. fujikuroi* were 40 µg/mL for $MIC_{90}$ values, while $MIC_{50}$ values ranged from 19.5 ± 2.5 to 21.5 ± 2.5 µg/mL. Similarly, the amendment of chitosan (shrimp shell) at 1% and 0.8% suppressed mycelial growth of *C. capsici*, a causal agent of anthracnose disease on chili, by 100% and 87.79%, respectively (Akter *et al.*, 2018).

This investigation indicated that commercial chitosan derived from shrimp waste produced by Vietnamese manufacturers showed broad-spectrum antifungal activity with a higher inhibition rate (%) compared to those reported in other studies. Moreover, chitosan displayed stability and durability in terms of quality, with its antifungal efficacy either maintaining or experiencing marginal reduction even under dilution. Likewise, chitosan possesses significant sustainability benefits at a competitive cost as it is derived from shrimp waste, thereby addressing environmental pollution concerns associated with the shrimp farming and processing industry (Parthiban *et al.*, 2017). However, antimicrobial activities of chitosan are dependent on its degree of deacetylation, molecular weight, and on specific strains of microorganisms, as indicated in previous publications, which underscores the necessity of employing precisely characterized chitosan to establish conclusive insights into its biological properties (Ke *et al.*, 2021).

## CONCLUSION

This *in vitro* study concluded that chitosan and its derivatives derived from shrimp waste byproducts exhibited the excellent ability to inhibit or directly control the development of pathogenic microbes isolated from Vietnamese agriculture. These investigations also indicated that chitosan from local shrimp waste may be recognized as a promising alternative to synthetic fungicides/antibiotics for controlling soil-borne pathogenic microorganisms thanks to its biofriendly and biodegradable properties, as well as the feasible and accessible application methods. For the extension application and escalation of the biological efficacy of chitosan, it is advisable to conduct more in-depth studies on characterizing chitosan. Additionally, exploring the synergistic combinations of chitosan with other natural compounds (such as extracts and essential oils) or chitosan with fungicide/antibiotics, is recommended. This approach aims to gradually reduce the overreliance on synthetic chemicals in agriculture.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTION

Conceptualization, investigation, material preparation and data collection by Long Dinh Tran. Methodology by LDT, VTT, TLP, QTN. Data curation by XBN and L DT. Manuscript writing by LDT. Review and editing by VTT, TTT, LDT, TDN. Supervision by VTT and TTT. All authors read and approved the final version of the manuscript.

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## DATA AVAILABILITY

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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