



Evaluation Of Freshwater Lobster (*Cherax Quadricarinatus*) Shells As A Green Bio-Fungicide Against *Fusarium* Sp

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Abstract

Fusarium species are very destructive soil-borne fungi that cause vascular wilt in several economically valuable crops. Nowadays, to overcome these outbreaks, the agricultural industry largely uses aggressive synthetic fungicides. In addition, the expanding aquaculture industry produces a lot of discarded crustacean shells. The aim of this research was to address both problems by extracting chitosan from the shells of freshwater lobsters (*Cherax quadricarinatus*) and exploring its efficacy as a bio-fungicide against the *Fusarium* sp. Freshwater lobster shells were subjected to conventional procedures for chemical extraction through demineralisation, deproteinization, and deacetylation. Following that, the purity of chitosan was determined through solubility analysis and acid-base titration. To evaluate the antifungal activities of chitosan, the Poisoned Food and Well Diffusion assays were performed, using different concentrations of chitosan (0.5% - 2.0%). These antifungal effects were measured by comparing them to the negative control (sterile distilled water) and the positive control (commercial fungicide Mancozeb). The results of the extraction process were highly satisfactory since high-purity chitosan with 96.6% Degree of Deacetylation (DD) was obtained, indicating high concentration of free amino groups. In Poisoned Food assay, the 2.0% chitosan solution had an extremely inhibition effect on fungal growth. Fungal proliferation was restricted up to 16.00 ± 1.73 mm, a 75% inhibition compared to 63.00 ± 2.65 mm of the control without any treatment. Statistically, the 2.0% concentration of natural product showed similar effectiveness as the chemical control. Meanwhile in Well Diffusion assay even though large molecule size limited the diffusion capacity of chitosan the research proved the practicality of freshwater lobster shells as environmentally friendly sources for obtaining potent antifungal agents.

Keywords: *Fusarium* spp., Freshwater lobster, Chitosan, Sustainable, Green bio-fungicide

1. Introduction

Fungal pathogens that cause substantial crop yield losses worldwide is currently posing an increasingly severe threat to agricultural production capacity in Malaysia (Fones et al., 2020). Among these, *Fusarium* spp. are highly destructive soil-borne fungi. It can infect a wide range of economically valuable crops, triggering aggressive diseases such as vascular wilt, root rot, and seedling blight. Crops that it can infest include bananas, tomatoes, maize, and oil palm seedlings (Dong et al., 2021; Zakaria, 2023). Preventing and controlling these pathogens is exceptionally challenging because their host range is extremely wide, complicating the implementation of comprehensive preventive measures. These fungi produce mycotoxins that harm crop and biological health and able to survive in soil matrices for extended periods. Even a small amount remains in the soil, infection can still occur during subsequent cultivation cycles (Nikitin et al., 2023; Shabeer et al., 2021). For a long time in the past, people relied on artificially synthesised chemical fungicides to prevent and control these crop diseases. Long-term dependence on this solution has triggered numerous concerns related to environmental safety, food security, and public health (Burandt et al., 2024; Rani et al., 2024; Zubrod et al., 2019). Chemical agents remaining in the environment first damage the originally rich biodiversity in soil and disrupt the natural soil ecosystem. They also flow with water into rivers, lakes, and seas, and gradually accumulate in the bodies of organisms in aquatic ecosystems a process professionally referred to as bioaccumulation. Coinciding with the problems of chemical pesticides is another unresolved situation which is the rapidly developing aquaculture industry in recent years has produced a large amount of otherwise valuable by-products, such as the shells of the freshwater lobster (*Cherax quadricarinatus*). These shells are often directly discarded as waste, failing to realise their value (Zhan et al., 2022). The shells of these crustaceans contain high concentrations of chitin, which is the core precursor material for preparing the biopolymer chitosan. Due to



its inherent advantage such as compatibility with biological environments, natural biodegradability, and intrinsic natural antibacterial properties chitosan is increasingly recognised as a natural bio-fungicide (de Asevedo et al., 2024; Elsabee & Abdou, 2013; Ke et al., 2021). Currently, commercial chitosan on the market is usually extracted from marine crustaceans, and the antifungal potential of chitosan specifically extracted from the shells of the freshwater lobster (*C. quadricarinatus*) against persistent pathogens such as *Fusarium* spp. remains a clear research gap, with no systematic research and verification having been conducted to date (Sayari et al., 2016). Developing and utilising this specific aquaculture waste stream is expected to combine two goals: solving the problem of disease management in agriculture and putting into practice the circular economy concept of turning waste into valuable resources. Based on this background, this study aimed to extract and detect chitosan from the shells of the freshwater lobster (*C. quadricarinatus*) and evaluate its efficacy as bio-fungicide against *Fusarium* sp.

2. Literature Review

2.1 *Fusarium* spp.: Biology and Impact

Fusarium spp. are ascomycete fungus with filamentous structures, which can be found in soils all over the world (Jackson et al., 2024). As plant pathogens, it ensures its own survival and spread by producing three distinct types of spores: microconidia, macroconidia, and chlamydospores that possess extremely strong viability and can survive for long periods. Relying on these spores, it can not only endure long periods of unsuitable environmental conditions to achieve long-term survival, but also quickly establish itself and spread on new host plants (Brodsky et al., 2023). Its invasion route in plants is well characterised. It first penetrates the plant's root system, then spreads along the vascular tissues responsible for transporting water and nutrients inside the plant and fully colonises the vascular system of the entire plant. This directly blocks the transport of nutrients and water in the plant, initially causing severe wilting symptoms, and eventually leading to the complete death of the entire plant (Moussaid et al., 2025). *Fusarium* causes extremely heavy losses to global agriculture. If its prevention and control are not effective enough, banana production in some banana plantations in Southeast Asian regions can drop by up to 60% (Xie et al., 2022).

2.2 Freshwater Lobster Industry in Malaysia

The cultivation scale of the freshwater lobster (*C. quadricarinatus*) has expanded rapidly within Malaysia and across the entire regional market. Its widespread adoption is entirely attributable to its rapid growth rate, high environmental adaptability, and substantial commercial value (Shehata et al., 2020). Leveraging these advantages, many locally adapted innovative aquaculture frameworks have been developed in Malaysia. For instance, the Freshwater Lobster Production-Green Aquaponics Perennial System (FLP-GAPS) in Sabah has integrated this farming model, specifically to support the local low-income B40 group (Rozita et al., 2022). The aquaculture industry for freshwater lobster delivers sound social and economic benefits, as it increases the income of low-income groups and drives local industries. However, after the harvested freshwater lobster are processed, a large and substantial amount of unused freshwater lobster shell waste is generated (Zhan et al., 2022). Reusing these otherwise wasted shells to produce high-value raw materials for agricultural production perfectly aligns with the current concepts of circular economy and sustainable development that are being promoted (Dong et al., 2024).

2.3 Antifungal Mechanisms of Chitosan

Chitosan is a substance processed from chitin as a raw material through deacetylation. Its antifungal effect is fundamentally tied to its inherent polycationic properties. The positively charged amino groups on its molecules bind to negatively charged components on the surface of fungal cells, and this binding disrupts the complete structure of the fungal cell membrane, causing the intracellular contents to leak out (Posnanski et al., 2023). In addition to damaging cell membranes, chitosan can directly penetrate the fungal cell wall to enter the cell interior. It inhibits the activity of various enzymes essential for fungal survival and disrupts the entire process of fungal RNA and DNA synthesis. The combined effect of these two actions can greatly restrict the germination of fungal spores as well as the expansion and growth of hyphae.

3. Methodology

3.1 Study Area and Sampling Design

The shells of the freshwater lobster (*C. quadricarinatus*) used in this experiment were collected from restaurants that specialise in aquaculture products. The collected shells were repeatedly rinsed to completely wash off all residual tissues such as remaining shrimp meat and fascia attached to the shells, to prevent interference with subsequent experiments. The cleaned shells were laid flat in a ventilated area to air-dry naturally for seven days. Once the shells desiccated and became brittle, it was ground into fine powder using a mechanical grinder to facilitate the subsequent chemical extraction process (Hosney et al., 2022; Kosma et al., 2022).



3.2 Extraction and Detection of Chitosan

Chitosan was extracted through a three-step chemical process consisting of demineralisation, deproteinization, and deacetylation (Rinaudo, 2006; Younes & Rinaudo, 2015). Each step was critical in isolating and converting chitin into chitosan with minimal contamination and high yield.

Demineralisation: The shell powder was soaked in hydrochloric acid (HCl) with a concentration of 1.0–2.0 M and left to stand at room temperature for 1-2 hours to completely remove the calcium carbonate in the shell powder. This step was adapted from Kanani et al. (2023), Sari et al. (2024) and Zhang et al. (2018).

Deproteinization: The samples that have completed the demineralisation treatment were placed in sodium hydroxide (NaOH) with a concentration of 1.0–2.0 M and treated at a temperature of 60-90°C for 1-2 hours to break the peptide bonds in the samples and isolate crude chitin. This procedure followed the method of Zhang et al. (2018).

Deacetylation: The obtained crude chitin was placed in NaOH with a concentration of 40-50% and treated at a temperature of 90-100°C for 2-4 hours to break the acetyl bonds in the material. This step was based on the of Novikov et al. (2023). To confirm that the degree of deacetylation (DD), the testing adopts the acid-base titration method. Hydrochloride acid (HCL) was added with a concentration of 0.1 M to complete the initial titration step, subsequently NaOH with a concentration of 0.1 M was used for back titration. During the entire titration process, methyl orange was used as an indicator to determine the titration end point. This testing method was adapted from Dutta & Priyanka (2022).

The degree of deacetylation DD% in percentage was calculated with the formula:

$$\%DD = \frac{(V1 - V2) \times 16}{M \times 9.94} \times 100$$

where V1 is the volume of NaOH consumed in the blank experiment, with the unit of mL; V2 is the Volume of NaOH consumed by the chitosan sample, with the same unit of mL; 16 is the equivalent weight of the amino group (-NH₂); M is the weight of the chitosan sample, with the unit of g; 9.94 is the theoretical amine content coefficient.

3.3 In Vitro Antifungal Assay

The chitosan solution was prepared by weighing the corresponding weight of the chitosan polymer and directly dissolved it in an acetic acid solution with a volume fraction of 1%, yielding final concentrations of 0.5%, 1.0%, 1.5% and 2.0% respectively. This preparation method was adapted from do Amaral Sobral et al. (2022), Melro et al. (2020) and Posnanski et al. (2023). The antifungal activities of the extracted chitosan were assessed using Poison Food assay. The test fungus *Fusarium* sp. was pre-cultured on Potato Dextrose Agar (PDA) medium and left to grow for 7 days at room temperature (28 ± 1°C). To prepare the poisoned, the chitosan solution was mixed into the still-melted, un-solidified liquid PDA medium under sterile conditions, stirred evenly and poured into petri dishes. Once the medium solidified completely, a 6-mm-diameter *Fusarium* sp. mycelial plug was placed at the centre of the petri dishes to complete the inoculation, following the method describe by Wan Yusof et al., 2024. The antifungal activities were further assessed using the Well Diffusion assay. Pre-prepared PDA petri dishes were punched with a sterile cork borer to create 6-mm-diameter wells at an equal distance from the fungal plugs placed in advance at the centre of the plate. Afterwards, 100 microliters of the test chitosan solution were transferred into each well, as described by Erhonyota et al., 2023. Control benchmarks were established for all tested treatment groups to compare the effects. The negative control used sterile distilled water, and the positive control used commercially available standard-concentration Mancozeb. The samples of all treatment groups and control groups were incubated in room temperature (28±1°C). After 7 days, the radial growth of the fungus in each sample was measured.

3.4 Experimental Design and Statistical Analysis

The study employed a Completely Randomised Design (CRD) with three replications per treatment. Data were expressed as mean ± standard deviation (SD). Results were analysed utilising IBM SPSS Statistics 27 via one-way Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at a significance threshold of $p < 0.05$ to differentiate the treatment means (Wan Yusof et al., 2024).

4. Results and Discussion

4.1 Chitosan Extraction and Detection

The extraction process of chitosan was successfully done through gradual steps and yielded a white-coloured powder with the same appearance as high-grade commercial biopolymers. Furthermore, the powder was shown to be completely soluble in 1% acetic acid solution, confirming the structural transformation from chitin to chitosan. Also, the determination of DD through acid-base titration indicated a value of about 96.6%. This is an outstanding achievement since DD value exceeding 90% is regarded as a milestone in this field of industry. The presence of high DD indicates that the chitosan derived from *C. quadricarinatus* has a high amount of free amino groups (-NH₂). Once dissolved in a water-based medium, these groups take on a positive charge. As Melro et al. (2020) pointed out, these positively charged



sites are the crucial active groups that allow the polymer to interact with other molecules and drive its antifungal properties.

4.2 Antifungal Activity: Poisoned Food Assay

The Poisoned Food assay made it obvious that the chitosan derived from *C. quadricarinatus* had excellent antifungal activity when applied directly. One-way ANOVA indicated highly significant variations between the treatment groups ($p < 0.001$). After a seven-day incubation period, the fungi present in the negative control samples had been able to grow quickly and without any obstacles, forming a colony that was 63.00 ± 2.65 mm in size. On the other hand, by applying chitosan to the agar, the fungi were significantly inhibited from growing depending on the concentrations used. At 2.0% concentration, the inhibition proved to be very efficient, with the fungi being capable of growing only 16.00 ± 1.73 mm. According to traditional metrics, this resulted in the strong Percentage of Inhibition of Radial Growth (PIRG) of 74.6% (Zhan et al., 2022). The Duncan's Multiple Range Test (DMRT) put the treatment group of 2.0% chitosan into the same statistical category with the positive control (Mancozeb), that limited the growth of the fungi to 11.00 mm. It can be stated that in its 2.0% concentration, chitosan shows the similar level of activity to the conventional synthetic fungicides when being used in solid culture media (Zhang et al., 2018). The antifungal activity of chitosan depends on its polycationic property, when it has many positively charged amino groups and binds to the negatively charged cell membranes of the *Fusarium* sp. This damages the structure of the membrane, thus causing the leakage of cellular contents and eventually kills the fungus (Erhonyota et al., 2023; Fones et al., 2020).

4.3 Antifungal Activity: Well Diffusion Assay

Based on the findings from the Well Diffusion assay, chitosan of 2.0% concentration exhibited notable significant growth inhibition ($p < 0.001$) of *Fusarium* sp. of 40.33 ± 0.58 mm, compared to Mancozeb at 34.33 mm. However, the percentage of inhibition of the former is much smaller than that of the latter obtained through Poison Food assay. It is clearly visible from the above information that the efficacy of chitosan becomes ineffective as it fails to diffuse itself passively in the dense agar medium. The phenomenon is explained by the well-known physical constraints of chitosan based on crustacean due to high molecular weight of chitosan (Burandt et al., 2024; Zakaria, 2023). Nevertheless, regardless of this restriction, the contact efficacy of chitosan shows that the efficient concentration of 2.0% is possible.

5. Conclusion and Recommendations

This study demonstrated that aquaculture waste can be converted into a valuable agricultural resource. This involved the successful extraction of chitosan having a degree of deacetylation of 96.6% from freshwater lobster shells. The antifungal activity against *Fusarium* sp. demonstrated an effective inhibition on the growth of the fungi. Interestingly, the Food Poison assay demonstrated that the 2.0% solution effectively inhibited 74.6% of fungal growth, which is similar to that of Mancozeb a commercial synthetic fungicide that are widely used to manage plant diseases. Future research works must include *in vivo* studies to ascertain its effectiveness in real-life situations. Moreover, future studies may include the development of nano-chitosan, which can increase its solubility in suitable solvents.

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