# Inhibition of Citrus Green Mold by Chitosan Obtained from Shells of Mantis Shrimp (*Oratosquilla nepa*)

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## Abstract

Chitosan is aminoglucopyranans consisted of $\beta$-(1,4)-linked glucosamine units together with *N*-acetylglucosamine units. It is present in the cell walls or fungi, green algae and in the exoskeleton of crustaceans. A novel procedure for preparing chitosan from the shells of mantis shrimp (*Oratosquilla nepa*) was developed. The procedure involves three steps composing demineralization, deproteinzation and deacetylation (immersed in 40% NaOH) processes. The obtained chitosan was characterized using Fourier transform infrared spectroscopy (FTIR). Samples of white chitosan with degrees of deacetylation (DD) as 96.20% were obtained, which analyzed by FTIR. *In-vitro* assay, the effect of 0.10% (w/v) of chitosan on *Penicillium digitatum* growth that cause postharvest disease in citrus fruit. The results indicated that it can against this fungus, the values of inhibition were significant complete as 99%$\pm$0.40.

**Keywords:** chitosan, *Oratosquilla nepa*, degrees of deacetylation, *Penicillium digitatum*

## Introduction

Chitosan is a natural, biodegradable, non-toxicity. The structure of chitosan is a linear polysaccharide which consisted of $\beta$-(1,4)-linked glucosamine units together with *N*-acetylglucosamine units. It is soluble in dilute solutions of various organic and inorganic acids (pH<6), due to the protonation of its amino groups [1]. The OH and $NH_2$ functionalities in chitosan's structure allow the preparation of diverse derivatives with improved properties for specific applications. Chitosan is used in a wide range such as the food industry, waste water treatment, cosmetics, medicine, pharmacy and agriculture [2,3,4]. For the agriculture, chitosan has been exhibited to be useful as well as increasing crop yields. Moreover, chitosan is associated with its fungicidal properties inhibit postharvest fungi such as *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* and *Penicillium digitatum* [5]. Green mold, caused by *P. digitatum* is important postharvest fungi causing severe diseases through injuries during harvesting, transportation and storage of citrus fruits resulting in economic losses [6]. The aim of this study was to synthesize chitosan from the shells of mantis shrimp (*Oratosquilla nepa*) and determine the effect of chitosan on mycelial growth of *P. digitatum*.

## Material and Methods

### *Chemicals*

The organic solvents were used in the experiments for analytical grade and purchased from Merck, Thailand.

### *Samples preparation*

Mantis shrimp (*O. nepa*) shells were obtained from a byproduct of the fishing industry, southern of Thailand. Firstly, the shells were washed by tap water several times and dried in a hot-air oven. After that they were homogenized in a blender into small sized pieces and kept frozen until used.

### *Chitosan production*

Chitosan was synthesized by three steps which composing demineralization, deproteinzation and deacetylation. Firstly, Demineralization was carried out by adding 1 L of 2 M HCl to 50 g of mantis shrimp shells. The reaction proceeded at room temperature for 2 h under agitation at 250 rpm. After that, they were filtrated and washed with distilled water until neutral pH. They were bleached by immersing in ethanol for 2 h and dried in an oven at 80 °C. For Deproteinization, deprotein was carried out by adding 2 M NaOH. The reaction was performed at 55 °C for 2 h then it was filtrated and washed with distilled water until neutral pH. After that, it was immersed in ethanol for 2 h for bleaching, and the resulting chitin was dried in an oven at 100 °C for 1 h. Finally, deacetylation of chitin was carried out by reacting chitin with 40%(w/v) NaOH. The temperature of the mixture was increased to 100 °C for 2 h with agitation at 250 rpm. The resulting chitosan was filtrated and washed with distilled water until neutral pH and then dried in an oven at 60 °C for 4 h.

### *Measuring the degree of deacetylation (DD)*

The degree of deacetylation of chitosan was determined by Fourier Transform Infrared Spectroscopy (FTIR). FTIR spectra were recorded at room temperature using a 400 Perkin Elmer spectrometer (Perkin-Dlmer, Norwalk, CA, USA) from 4000 $cm^{-1}$ to 400 $cm^{-1}$. Sample was dried and ground with KBr. DD was calculated from the spectra using formula [7].

$$DD\% = 100 - \frac{A1655}{A3450}\ x\ 115$$

### *Penicillium digitatum and chitosan preparation*

*P. digitatum* was isolated from citrus fruit rot, kindly provided by the Prince of Songkhla University, Thailand, and grown in potato dextrose agar (PDA) plate at room temperature for 7 days. For chitosan, a stock solution of chitosan was prepared in 1% (v/v) acetic acid with pH5.6, stirring at 150 rpm for 24 h at room temperature, then it was autoclaved at 121 °C for 15 min. After that, the chitosan solution was mixed with the PDA medium until the final concentration at 0.1, 0.05 and 0.01% (v/v). Sterile distilled water of pH5.6 was used as a control.

### *Antifungal activity testing*

The effect of chitosan on *P. digitatum* growth was determined. The mycelial disc of *P. digitatum* was put on the center PDA medium amended with each chitosan (final concentration 0.1, 0.05 and 0.01% (v/v)). Plates were incubated at room temperature for 7 days. After that, the diameters of the fungal colonies were assayed by an equation of Gamliel and coworker [8], which presented as %inhibition. Each treatment was replicated using three plates, and experiment was performed three times.

$$\%\text{inhibition} = 100\text{-}[(R^2/r^2)100])$$

(when R is radial of the fungal colonies for control, r is radial of the fungal colonies for treatment)

### *Statistical analysis*

The data were performed by one-way analysis of variance (ANOVA) at $p<0.05$ Significant means were compared by Duncan's multiple range test using SPSS Statistics 17.0 softwere.

## Results and Discussion

### *Chitosan production*

Chitosan samples from shell of mantis shrimp were shown in Figure 1. They were obtained as a white powder after the demineralization and deproteinization steps that the results were similar with Antonino and coworker [9].

**Figure 1.** Chitosan from shell of mantis shrimp were synthesized by 40% (w/v) NaOH

### *Degree of Deacetylation*

The FTIR spectra of chitosan from the shells of mantis shrimp was shown in Figure 2. The results showed characteristic absorption bands similar during commercial chitosan and chitosan samples (Figure 2A and 2B) at 3450 $cm^{-1}$ which refers to O-H stretching, Aliphatic C-H stretching at 2925 $cm^{-1}$, N-H stretching (Amide I) at 1655 $cm^{-1}$, 1580 $cm^{-1}$ ($-NH_2$ bending), and 1320 $cm^{-1}$ (Amide III) [10]. The degree of deacetylation of chitosan samples from FTIR spectra was shown as 96.20% (Figure 2B). When DD was calculated from the spectra using formula [7].

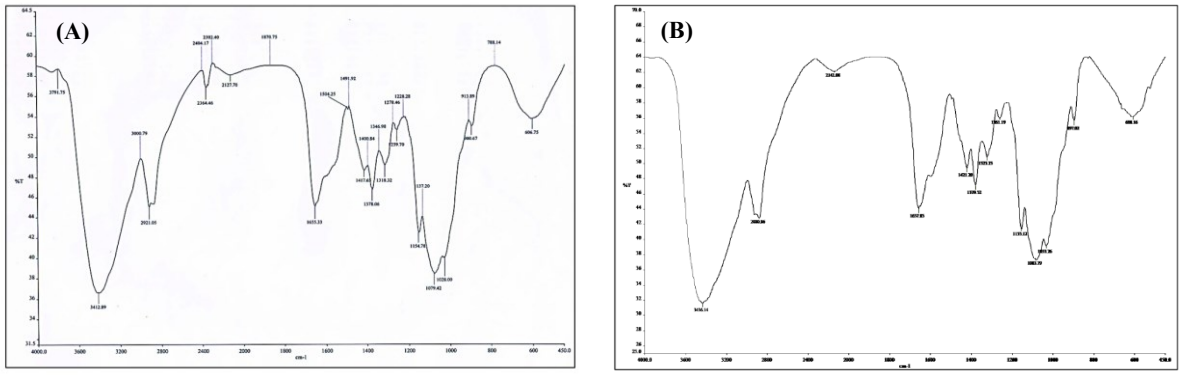


**Figure 2.** FTIR spectra of chitosan; (A) commercial chitosan, (B) chitosan samples

***Determination of chitosan antifungal activity***

The effect of 0.01%, 0.05% and 0.10% (w/v) chitosan on *P. digitatum* growth showed in Figure 3 and Table 1. The results exhibited the concentration of 0.10% (w/v) chitosan was more effective against fungi *P. digitatum* (Figure 3D) when compared to the control (Figure 3A), including the concentration of 0.01% (Figure 3B) and 0.05% (w/v) chitosan (Figure 3C). The values of inhibition were significant complete as 99% (Table 1) while the concentration of 0.01% and 0.05% (w/v) chitosan as 62% and 96%, respectively. The inhibitory effect of chitosan was calculated when the control plate was incubated for 7 days. There is strong evidence that the fungal mycelium growth can inhibit by using chitosan. The concentration of 3% (w/v) chitosan can completely inhibit the fungi *F. oxysporum*, *Rhizopus stolonifer*, *C. gloeosporioid*es and *P. digitatum* [11,12].

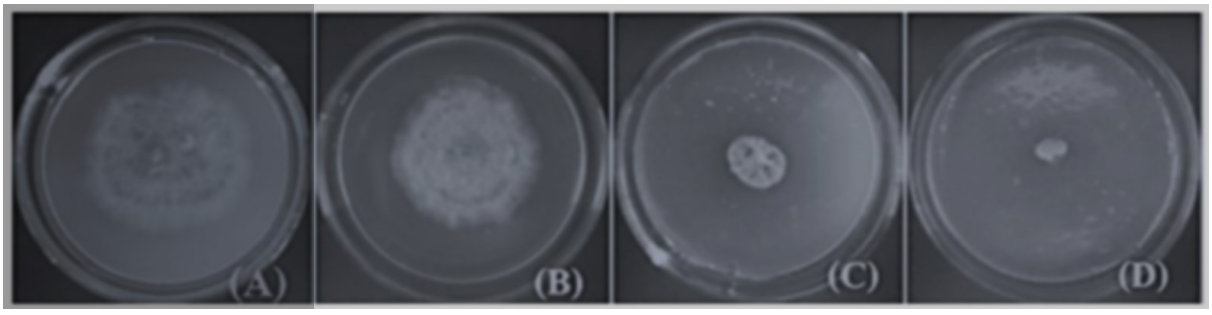


**Figure 3.** Effect of each chitosan to inhibit *P. digitatum* for 7 days; (A) control, (B) 0.01%(w/v) chitosan, (C) 0.05%(w/v) chitosan and (D) 0.10%(w/v) chitosan

**Table 1.** Effect of each chitosan for antifungal activity

| sample | % inhibition±SD |
|---|---|
| 0.01%(w/v) chitosan | $62^{c}$±0.98 |
| 0.05%(w/v) chitosan | $96^{b}$±0.79 |
| 0.10%(w/v) chitosan | $99^{a}$±0.40 |

## Conclusions

In the summary, chitosan was synthesized from shells of mantis shrimp (*O. nepa*) as a white powder. The degree of deacetylation (DD) is an important property of chitosan to analyze the way of application of the biopolymer. Chitosan was partially deacetylated and obtained samples were characterized using FTIR. It is noticed that the acetyl group can contribute to the formation of hydrogen bonds that can stabilize the crystalline structure. In addition, it is found that chitosan sample properties suitable for agricultural applications are obtained.

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