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Preparation and Characterization of Chitosan obtained from Pacific White Shrimp Shells and its *in vitro* Antifungal Activity

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In this article, a method for the processing of chitosan from Pacific white shrimp shells is developed which involves three steps *viz.* demineralization, deproteinization, and deacetylation. The samples of chitosan with more than 90% degree of deacetylation (DD%) were obtained by FTIR. This indicated that the current processing method of shrimp shells was beneficial for chitosan production. The morphology of chitosan sample was determined using scanning electron microscopy (SEM). X-ray diffraction (XRD) patterns exhibited two peaks of crystalline character approximately at 10º and 20º (2θ). The effect of 0.1% (w/v) of chitosan on the growth of *Penicillium digitatum* was tested by an *in vitro* assay and the results showed an almost complete inhibition (98% ± 0.56).

Keywords: Chitosan, Pacific white shrimp, Degrees of deacetylation, *Penicillium digitatum*.

INTRODUCTION

The green mold of citrus fruit, caused by *Penicillium digitatum* Sacc., is one of the most economically important postharvest diseases of citrus fruits. The fungus attacks the citrus fruits only through injuries from where nutrients and moisture are obtainable, and this provokes spore germination and infection and causes serious postharvest losses during harvesting, transportation and storage or marketing [1,2]. Fungicides, such as imazalil, thiabendazole and *o*-phenylphenol, have been used to control *P. digitatum* induced fruit decay in the packing house [3-5]. However, repeated use of fungicide toxicity affects the environment and human health, including the development of fungicide resistance by pathogens.

Chitosan is a deacetylated derivative of chitin, a copolymer that consists of a group of heteropolysaccharides comprising of β-1,4-linked D-glucosamine (deacetylated unit) and *N*-acetyl D-glucosamine (acetylated unit) residues. Chitosan is the most abundant basic biopolymer and is structurally similar to cellulose that consists of only one monomer of glucose. It is characterized by non-toxicity, biocompatibility and biodegradability [6]. Chitosan is a natural polysaccharide commonly found in the outer shell of crustaceans (shrimps, crayfishes and crabs), fungal cell walls, green algae, and the cuticles of arachnids and insects. The major compounds of crustacean shells are 15-40% of chitin, 20-40% of protein, 20-50% of calcium and magnesium carbonate, including lipid, astaxanthin and other minerals [7]. Chitosan is a promising molecule with a various applications in cosmetics [8], medicine [9,10], pharmacy [11], agriculture [12], agricultural product preservation [13], the food industry [2], wastewater treatment [14] and other industrial applications. The mechanism for the antimicrobial action exhibited by chitosan indicates that the positive charge is generated by the protonation of the free amino group at the acidic pH. Polycationic chitosan can react with the negatively-charged fungal cell membrane components (proteins, phospholipids), which interfere with the metabolism of fungal cell and normal growth [15]. The polycationic properties allows it to interact with polyanions producing polyelectrolyte complexes [16]. It has a great film forming capability and hence, can also be prepared as micro/nanoparticles, hydrogels, fibers and films [17]. The functional groups of -OH and -NH_2 in the chitosan structure allow for the preparation of a variety of derivatives with improved properties for specific applications. The amino groups have the capacity to react with many of anionic groups on the cell wall surface of yeast, therefore producing an imper-

vious layer around the cell. Due to the film property formation, chitosan may acts as a barrier and reduce the level of growth of the pathogens [18].

Pacific white shrimp (*Litopenaeus vannamei* Boone) species are available in large amount in the breeding areas in southern Thailand, wherein the sources of seafood generate a considerable amount of discards. This waste consist of chitin and protein rich, which can be evaluated if the extraction methods of these shrimp shell components is well designed and implemented. In the present work, a processing method is developed for the preparation of chitosan from shrimp shell. The chitosan was characterized using Fourier transform infrared spectrometry (FTIR), scanning electron microscopy (SEM) and X-ray diffraction (XRD). After that chitosan was measured for its action against *P. digitatum* Sacc. that causes disease in the citrus fruit.

EXPERIMENTAL

Sample preparation: Pacific white shrimp (*L. vannamei* Boone) shells were obtained from the Nakhon Si Thammarat province, Thailand. Firstly, the shells were washed several times with tap water to remove impurities and then dried in a hot-air oven at 100 ºC for 2 h. For the production of chitin and chitosan, the shells were homogenized in a blender into small pieces and placed in refrigerator until used.

Demineralization: Demineralization was performed by adding 1 L of 2 M HCl to 50 g of the shrimp shells. The reaction carried out at room temperature for 2 h along with agitation at 250 rpm. Later, the demineralized shrimp shells were filtered and washed with distilled water several times until neutral pH was achieved. They were bleached by immersing in ethanol for 2 h and dried in an oven at 80 ºC.

Deproteinization: Deproteinization was carried out by adding 2 M NaOH to the dried, demineralized shells at a ratio of 1:20 (g/mL). The reaction was performed at 55 ºC for 2 h; the shrimp shells were filtered and washed with distilled water until neutral pH. Later, they were soaked in ethanol for 2 h for bleaching and the resulting chitin was dried in an oven at 100 ºC for 1 h.

Chitosan production: Deacetylation is the process of converting chitin to chitosan by the removal of acetyl groups. This process was repeated twice. The chitin was treated in two ways: by reacting with 40% (w/v) NaOH to give chitosan I (CHT- I) or with 60% (w/v) NaOH to give chitosan II (CHT-II) at a ratio of 1:20 (g/mL). The temperature of the mixture was increased to 100 ºC for 2 h to allow deacetylation by agitation at 250 rpm. The resulting chitosan was filtered and washed with distilled water until neutral pH and then dried in an oven at 60 ºC for 4 h.

Degree of deacetylation: The degree of deacetylation (DD) was measured by Fourier transform infrared spectroscopy (FTIR). FTIR spectra were obtained from a Perkin-Elmer spectrometer (Perkin-Elmer, USA) ranging from 4000 to 400 cm^{-1} at room temperature using KBr pallets. Degree of deacetylation (DD) was determined [19] from the FTIR spectra using the following formula:

$$\text{Degree of deacetylation} = 100 - \frac{A_{1655}}{A_{3450}} \times 115$$

where, A_{1655} and A_{3450} present the absorbance of chitosan at 1655 and 3450 cm^{-1}, respectively.

SEM analysis: The morphology of the chitosan samples was evaluated using a scanning electron microscope (SEM) model TM-1000 (Hitachi, Tokyo, Japan) performed at an acceleration voltage of 15 kV. The microscope was installed with an energy dispersive spectroscopy (EDS) detector (Oxford Instruments, Oxford, UK). The chitosan samples for SEM were proceeded by depositing onto a carbon tape.

X-ray diffraction: The crystalline nature of the chitosan samples was recorded by wide-angle X-ray diffraction (WAXD) analysis using an XRD 7000 Shimadzu (Shimadzu, Kyoto, Japan) at room temperature. The diffractometer was proceeded with Cu$K\alpha$ radiation ($\lambda = 0.15418$ nm) from a broad focus Cu tube operated at 40 kV and 40 mA; it was applied to the sample for measurement. Diffraction patterns were determined over a 2θ range of 5º-100º in the continuous mode.

***Penicillium digitatum* and chitosan preparation:** *P. digitatum* was isolated from the fruit rot of citrus, which was kindly provided by the Prince of Songkla University, Thailand, and grown on potato dextrose agar (PDA) plates at 25 ºC for a week. A stock solution of chitosan was dissolved in 1% (v/v) acetic acid, adjusted to pH 5.6 with NaOH, stirred (150 rpm) at room temperature for 24 h, and then autoclaved at 121 ºC for 15 min. The sterile distilled water of pH 5.6 was used as the control. After that the chitosan solution was mixed with the PDA medium to prepare final concentrations of 0.1, 0.05 and 0.01% (w/v).

Effect of chitosan on *in vitro* mycelial growth: The effect of different concentrations of chitosan on *P. digitatum* growth was determined by inoculating a mycelial disc of 4 mm diameter with the edge of a 5-day-old colony of *P. digitatum* onto the center of PDA medium amended with different chitosan concentrations (final concentration 0.1, 0.05 and 0.01% (w/v)). The plates were incubated at 25 ºC. The diameters of the fungal colonies were determined when the control PDA medium was completely spread with the mycelium of *P. digitatum.* The percent inhibition was calculated according to the eqn. 2 as reported by Gamliel *et al*. [20]. Each treatment was replicated using three plates and the experiment was proceeded thrice.

$$\text{Inhibition } (\%) = 100 - \left(\frac{R^2}{r^2} \times 100 \right) \quad (2)$$

where R is the radius of the fungal colonies in control, r is the radius of the fungal colonies in treated samples)

Statistical analysis: The data were examined by one-way analysis of variance (ANOVA) at $p < 0.05$. The mean values were compared by using SPSS Statistics 17.0 software (Duncan's multiple range test).

RESULTS AND DISCUSSION

Chitosan was easily obtained from shrimp shell waste, particularly from the Pacific white shrimp shells. Shrimp shell powder, light yellow in colour, was dried and finely blended. After the demineralization and deproteinization steps, it was turned into a white powder. Shrimp shell waste is the richest

source of chitin and a major source among the crustaceans that can be processed into chitin.

For chitosan processing by deacetylation, chitosan samples from the shell shrimp waste were prepared by different reaction conditions. Chitosan samples were obtained as a white powder similar to that obtained by Antonino *et al*. [21].

FTIR spectral studies: The FTIR spectra (Fig. 1) showed that the structure of CHT-I and CHT-II was similar to that of the commercial chitosan (control) and various characteristic peaks were observed. The characteristic absorption bands at 3500-3000 cm^{-1} that refer to O-H stretching, aliphatic C-H stretching bands around 2925 cm^{-1}, N-H stretching (amide-I) bands around 1655 cm^{-1}, 1580 cm^{-1} (-NH_2 bending) and 1320 cm^{-1} (amide III) were observed. The absorption peaks observed around 1160 cm^{-1} (anti-symmetric stretching of C-O-C bridge) and 1082 cm^{-1} (skeletal vibrations involving the C-O stretching are characteristic of its saccharide structure) correlated with the results of Barbosa *et al*. [22].

Degree of deacetylation: The degree of deacetylation of chitosan samples from the FTIR spectra were evaluated using eqn. 1. The degrees of deacetylation were 94.23% ±0.01 and 92.45% ±0.02 when chitin samples were converted to chitosan by the removal of the acetyl group with 40% (w/v) NaOH (CHT- I) and 60% (w/v) NaOH (CHT- II), respectively. The CHT-I sample for the analysis by SEM, EDS and antifungal activity was selected as its degree of deacetylation was higher than that of CHT-II. The degree of deacetylation (DD) of chitosan, defined as the ratio of acetylglucosamine units in the polymer, depends on the deacetylation conditions. It was reported [23] that the chitosan samples obtained from the Chilean freshwater crab (*Aegla cholchol*) exoskeleton possessed between 4% and 15% of *N*-acetylation (DA). The degree of acetylation of chitosan samples from the shrimp shells was below 9% as calculated by FTIR and UV-first derivative spectroscopy [21]. In addition, chitosan samples with different degrees of deacetylation (DD) were assayed by thermogravimetry coupled to infrared spectroscopy (TG-FTIR) to test the effect of DD on thermal analysis. DD values of chitosan were measured by ^{1}H NMR and presented as 98%, 87%, and 71% [22].

SEM studies: The morphological features of the chitosan prepared from shrimp shell waste were determined by SEM (Fig. 2). The micrographs of CHT-I showed a heterogeneous and rough surface with straps. At a higher magnification, the fibrous nature of the material was exhibited (Fig. 2b and 2c). The EDS analysis of CHT-I indicated that it consisted of C (62.5%), O (37.0%), and Ca (0.5%) by weight (Fig. 2d). After demineralization, marked changes were observed on the surface material. The chitosan obtained from shrimp shells exhibited a heterogeneous morphology and fibrous structure. The EDS analysis revealed the absence of Ca in the demineralized shells [21]. The crustacean shell mineral content varies widely for each species. The EDS analysis of the Chilean freshwater crab exoskeleton indicated the presence of oxygen, carbon, calcium, and chlorine as main components in the shell surface of the crab, as well as potassium, bromine, aluminum and copper in trace amounts [23].

XRD studies: The diffractogram of the chitosan sample is shown in Fig. 3. The XRD pattern of CHT-I exhibited two characteristic broad diffraction peaks at 2θ around 10º and 20º which were typical fingerprints of the semi-crystalline character of chitosan. Dey *et al*. [24] reported that the XRD pattern of chitosan obtained from waste prawn shells revealed characteristic wide diffraction peaks at 2θ around 9.63º and 20.53º, which are typical of semi-crystalline chitosan. The XRD patterns presented the characteristic peaks of chitosan obtained from shrimp shells at 10º and 20º in 2θ corresponding to the crystalline nature; the crystallinity index (CrI) of chitosan was around 40% [21].

Antifungal activity: The effect of different concentrations (0.01, 0.05 and 0.10% (w/v)) of CHT-I on *P. digitatum* growth is shown in Fig. 4. The results exhibited that the concentration of 0.10% (w/v) CHT-I was more effective against the *P. digitatum* (Fig. 4d) when compared to the control (Fig. 4a) and the concentrations of 0.01% (w/v) (Fig. 4b) and 0.05% (w/v) CHT-I (Fig. 4c). The inhibition was high (98% ± 0.56) at the concentration of 0.10% CHT-I, while 0.01% and 0.05% (w/v) CHT-I showed low percentage inhibition as 54% and 91%, respectively (Table-1). There is strong evidence that the fungal mycelial growth can be inhibited by chitosan. Two mechanisms have been proposed for chitosan to inhibit the microbial cells. First, the polycationic nature (positive charge) of chitosan interferes with the bacterial metabolism by electrostatic stacking (negative charge) at the cellular surface [25,26]. Second, the antimicrobial activities of chitosan depend on the level of the degree of deacetylation (DD). Chitosan with a higher degree of deacetylation showed higher antimicrobial activity [27]. The other mechanism involves the blocking of RNA transcription from DNA by adsorption of the penetrated chitosan to DNA molecules. A sample of 3.0% (w/v) chitosan can completely inhibit the fungi *F. oxysporum*, *R. stolonifer*, *P. digitatum* and *Colletotrichum gloeosporioides* [28,29]. In addition, the

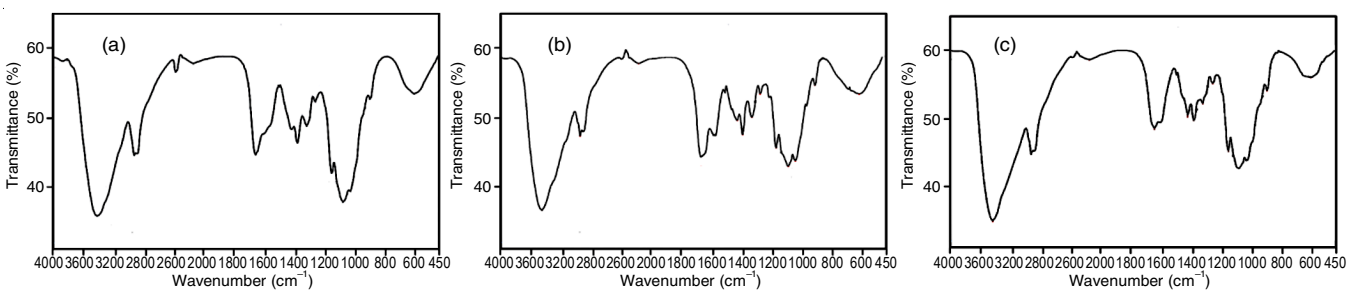

Fig. 1. FTIR spectra of commercial chitosan (a), CHT-I (b) and CHT-II (c)

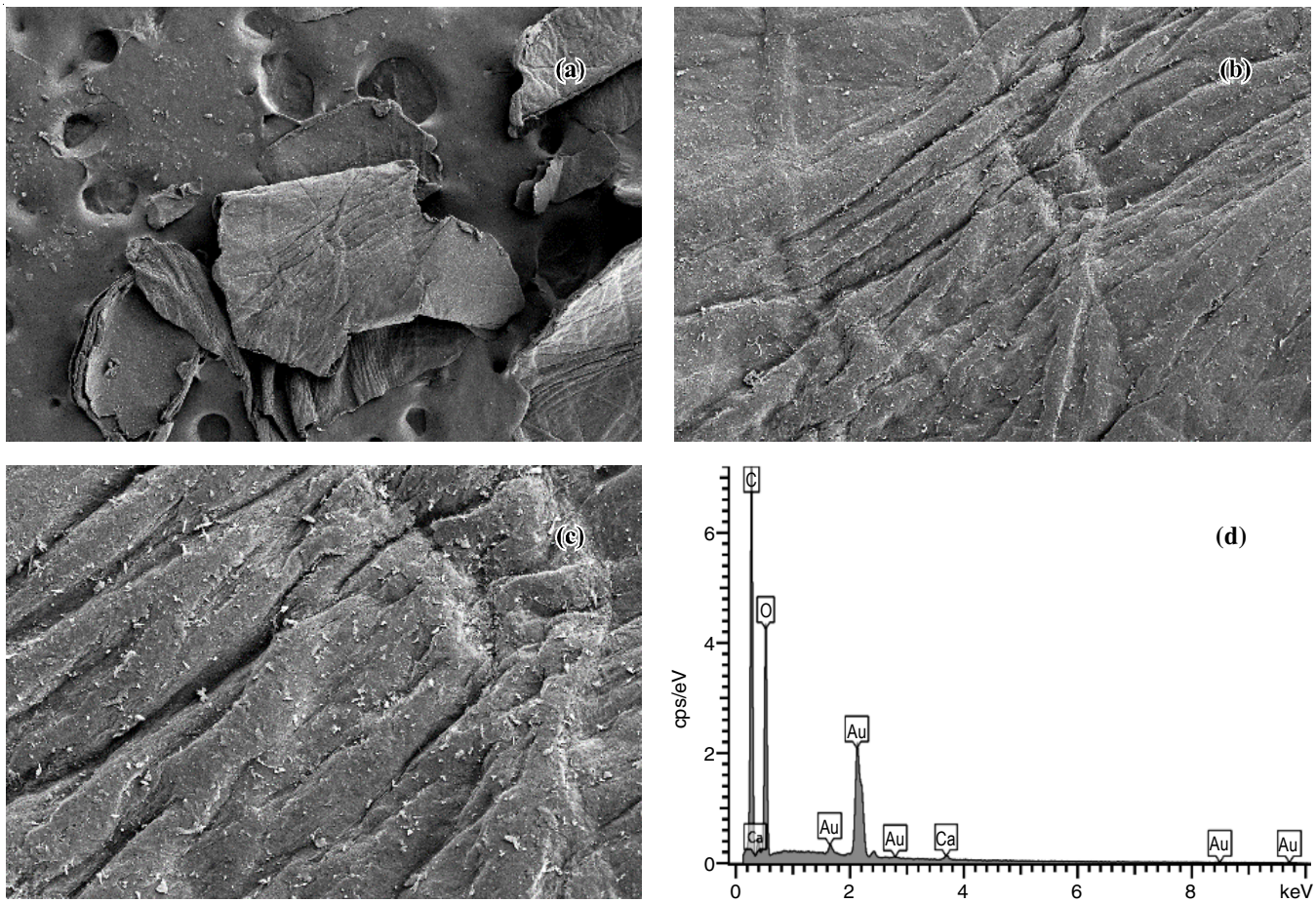

Fig. 2. SEM images of CHT-I at different levels of magnification after deacetylation; 50× (a), 200× (b), 500× (c) and EDS image (d)

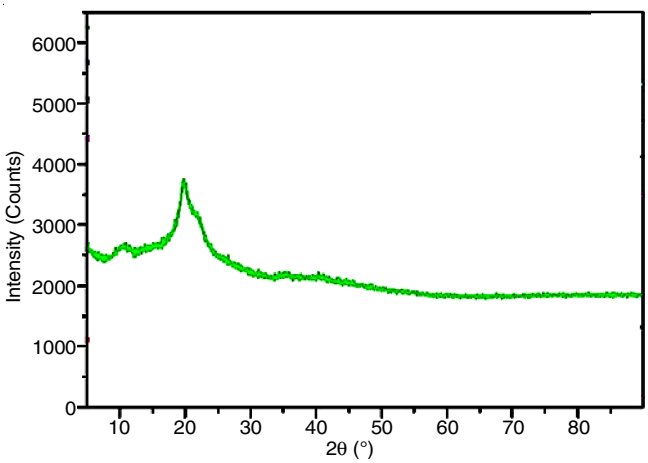

Fig. 3. XRD spectrum of CHT-I

TABLE-1
EFFECT OF EACH CHITOSAN FOR ANTIFUNGAL ACTIVITY

Sample	% Inhibition ± SD
0.01% (w/v) CHT-I	$54^{c} \pm 0.98$
0.05% (w/v) CHT-I	$91^{b} \pm 0.50$
0.10% (w/v) CHT-I	$98^{a} \pm 0.56$

same concentration of 3.0% (w/v), when used in chitosan films blended with silver nanoparticles, showed higher antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Pseudomonas aeruginosa* [30]. Chitosan with a lower degree of acetylation (DA 4%) and higher molecular weight presented the lowest minimum inhibitory concentration (MIC) values as evidenced by the highest antifungal activity toward *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* [23]. Moreover, a sample of 0.2% (w/v) chitosan, when used as a spray, activated defense responses in the rubber tree against *Phytophthora palmivora*. It has been reported that chitosan can reduce the disease severity in rubber tree through the up-regulation of ABA-biosynthesis genes and defense-related genes, which lead to the stimulation of defense-related proteins and increased level of abscisic acid in the rubber tree [31].

Conclusion

In this work, semi-crystalline chitosan was synthesized as a white powder from the Pacific white shrimp shells. The degree of deacetylation (DD) was above 90%. In addition, chitosan showed antifungal activity against *P. digitatum* at 98% ± 0.56.

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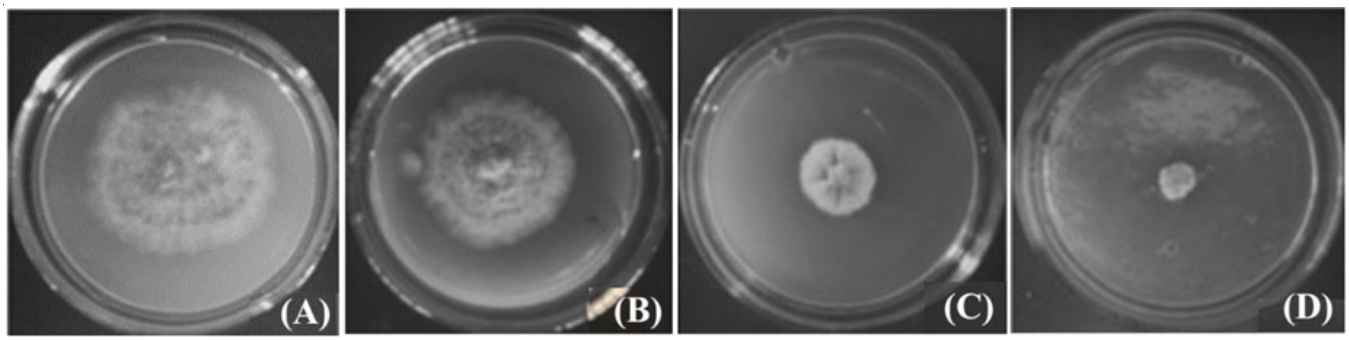

Fig. 4. Growth inhibition of *P. digitatum* by chitosan for 7 days; control (A), 0.01% (w/v) CHT-I (B), 0.05% (w/v) CHT-I (C) and 0.10% (w/v) CHT-I (D)

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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