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Preparation, characterization and Anti-fungal Activity of Biodegradable Polymer (chitosan) on Some Phytopathogenic Fungi

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ABSTRACT

Chitosan, a natural biopolymer is one of the most important derivatives of chitin, which is one of the major structural elements, that forms the exoskeleton of crustacean shrimps. The objective of the present study was to extract the chitosan from shrimp shells waste and then it was characterized by the Fourier Transform Infrared Spectroscopy, X-ray diffraction pattern, Scanning electron microscope analysis, the thermal properties, the degree of deacetylation and molecular weight was measured. Antifungal activity of chitosan was tested by the poisoned food technique against some phytopathogenic fungi. The results indicated that the percentage inhibition of mycelial growth increase with increasing concentrations of chitosan for all tested strains. It was clear that Macrophomina phaseolina; Fusarium oxysporum and Phytophthra sp showed strong sensitivity to chitosan and the EC_{50} values were 430.63, 566.23 and 677.22 ppm, respectively. Meanwhile, it displayed moderate high antifungal activity against the others. Chitosan could be used as a potential antifungal agent for the control of fungal plant diseases.

Keywords: Chitosan, biodegradable, antifungal activity, phytopathogenic fungi.

INTRODUCTION

Phytopathogenic organisms cause a wide spectrum of diseases in plants and include fungi, nematodes, bacteria, and viruses (Montesinos, 2003). Plant pathogenic fungi can cause severe reducing yields, lowering product quality or decreasing nutritional value of crops, and, sometimes, contaminating food with toxic compounds, which results in such dramatic economic losses in agriculture. (Kusuma et al., 2005; Bajapi et al., 2009; Wilson and Talbot, 2009; Gautam et al., 2011). Synthetic chemicals such as fungicides have been used for a long time and have greatly contributed in management of such losses (Spadaro and Gullino, 2004). The application of such chemicals has led to a number of environmental and health problems due to their residual toxicity, carcinogenicity, hormonal imbalance and spermatotoxicity (Kumar et al., 2007). Because of indiscriminate use, some microorganisms have developed resistance to most widely used synthetic fungitoxicants rendering them out of date (Wilson et al., 1997). Hence, there is a need to develop

new fungicides with improved performance as well as ecofriendly in nature. (Ben-Yeohshua and Mercier, 2005; Meng et al., 2008). At present, increasing interest is being devoted to the use of natural substances such as bioactive chitosan polymers. Chitosan is one of the most important derivatives of chitin, which is the second most abundant natural biopolymer found on earth after cellulose (No and Meyers 1989) and is a major component of the shells of crustaceans such as crabs and shrimps. Chitosan can be obtained by N-deacetylation of chitin. (Muzzarelli, 2009; Jayakumar et al., 2010). One of the most attractive features of chitosan is its antibacterial, antiviral, antifungal activity, antitumour and immune-enhancing effects. (Lin et al., 2009; Zhang et al., 2011). Recently the utilization of chitosan as a food preservative (Kumar, 2000), textile (El Tahlawy et al., 2005), waste water treatment (Chi and Cheng, 2006), paper finishing, photographic paper (Kumar, 2000) or adjuvant in agriculture to protect or stimulate the defense of different crops has increased. (Zhang et al. 2011; Jayakumar et al. 2011). Chitosan is Generally Recognized As Safe (GRAS) by the US FDA (2001). The main aim of the present study was to prepare chitosan from shrimp shells waste which are hazard and toxic for environment and evaluate the antifungal activity of the prepared chitosan against some economically important phytopathogenic fungi.

MATERIALS AND METHODS

Materials

Acetic acid, hydrochloric acid, oxalic acid, potassium permanganates, sodium chloride, potassium hydroxide, methanol, acetone and sodium hydroxide were purchased from Merck KGaA Darmstadt, Germany, and were used without further purification. Agar and Dextrose used in the biological activity test are products of El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt. Streptomycin Antibiotic is a product of El Nil Co. for Pharm. and Chem. Ind, Cairo, Egypt. Water used in all preparations obtained from water distillatory LABCONCO water PRO^{TM} PS LABCONCO Corporation, KANSAS City, Missouri 64132-USA.

Raw material

The shrimp shells were collected from sea food shops and washed under running water to remove soluble organics, adherent proteins and other impurities. Then it dried in oven at 70°C for 24 h or longer until completely dried shells were obtained.

Fungal strains used.

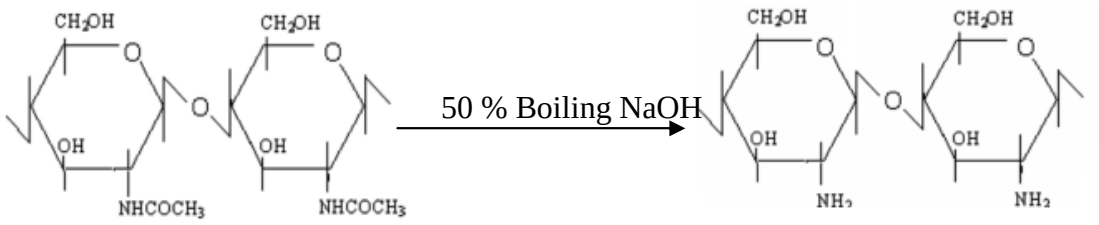
Phytopathogenic fungi were used in this study: *Fusarium Solani (F.Solani); Fusarium oxysporum (F.oxysporum); Phytophthra sp; macrophomina phaseolina (M. phaseolina); Alternaria Solani (A.Solani); phoma sp and Remularia sp.* They were provided by Fungicides, Bactericides and Nematicides Department, Central Agricultural Pesticides Laboratory (CAPL), Dokki, Giza, Egypt. The Fungi were regularly maintained on dextrose agar (PDA) media (200 g grated potato, 20 g dextrose, 15 g agar) (Atlas, 1995).

Methods

Preparation of Chitosan.

The chitinous material (shells of the shrimp) was decalcificated with 1.0 M HCl (3.0%w=v) at room temperature with constant stirring for 1.5 hours. The decalcified product was filtered, washed and dried, then deproteinized with 4% NaOH solution at 50°C with constant stirring for 5 hours. The deproteinized chitin was filtered and washed with de-ionized distilled water until the pH became neutral. It was dehydrated twice with methanol, and once with acetone, and dried. The dried chitin was added to boiling 0.1% potassium permanganate solution to remove the odor and to 15% oxalic acid solution to remove the color. The product chitin was filtered, washed with

distilled water and dried. The chitosan was prepared by adding the dried chitin into a three-necked flask containing a solution of 40% KOH. It was refluxed under nitrogen atmosphere at 135-140°C for 2 hours. The deacetylated chitin (chitosan) was filtered, washed with distilled water, and dried. (Abdeen, 2011; Abdeen, 2005).



CHITIN **CHITOSAN**

Fig 1: The preparation of chitosan by deactylation of chitin

Physicochemical characterization of prepared chitosan.

Degree of Deacetylation

The degree of deacetylation (D.D.) of the prepared chitosan was determined by using a Ati Mattson Fourier Transform Infra Red (FTIR) instrument with frequency of 4000-400cm^{-1}. The deacetylated chitin (chitosan) was subjected to infrared spectroscopy to calculate the degree of deactylation (D.D) %, by the relationship:

$$D.D = 100 - [(A1660/A3450) \times 100 / 1.33] (\%), \quad (1)$$

Where, A= absolute height of the absorption band of the amide group and hydroxyl group respectively (Moore and Roberts, 1980).

Molecular Weight Determination

Molecular weight is one of the significant characteristics that control the functional properties of chitosan. Viscosity is one of the simple techniques that is widely used for estimation of the molecular weights of polymers. The viscosity-average molecular weight was calculated using Mark-Houwink equation relating to intrinsic viscosity [Blair et al., 1987)

$$[\eta] = K_m M_v^a \quad (2)$$

where $K_m = 8.93 \times 10^{-4}$ and a = 0.716 at 25 °C are the empirical viscometer constants that are specific for a given polymer, solvent and temperature.

Degree of Swelling.

Swelling is the most significant characteristic of hydrogels and it reflects the affinity of the chemical structure of hydrogels for water and other surrounding fluids.

Preparation of Chitosan Gel Film.

Gel was prepared by dissolving 2 % (w/v) chitosan in1% (v/v) aqueous acetic acid with constant stirring at room temperature. The vicious solution prepared was filtered through a cheese cloth to remove any impurities and cast in petri dish to dry forming thin film. A known weight of the chitosan films was immersed in solutions of different pH (4,7) at 25°C and 37°C until the swelling equilibrium was reached. The films were removed, dried with absorbent paper to get rid of excess water and then weighed. The degree of swelling of these samples was calculated with the following equation:

DS= (m- m') / m' (3)

Where m and m' denote the weights of hydrogel sample and dried hydrogel sample, respectively (Ferrus and Pages, 1977).

X-ray diffraction (XRD) Study.

The prepared chitosan was characterized by x-ray diffraction (XRD) using an x-ray diffractomete Siemens (Berlin, Germany) D500 diffractometer with a back monochromator and a Cu anticathode.

Scanning electron microscopy testing

Phase morphology was studied using a JSM-T20 (JEOL, Tokyo, Japan) scanning electron microscope (SEM). For scanning electron observations, the surface of the sample was mounted on a standard specimen stub. A thin coating (~ 10^{-6} m) of gold was deposited into the sample surface and attached to the stub prior to SEM examination in the microscope to avoid electrostatic charging during examination

Thermal properties

Thermo-gravimetric analysis (TGA)

A thermal weight change analysis instrument (TGA) was used to measure the amount and rate of change in weight of the material as a function of increasing temperature or time, in a controlled atmosphere. All TGA spectra were recorded under a nitrogen atmosphere up to 600°C using a programmed rate of 10 °C/min. The percentage of weight loss was plotted against temperature.

Preparation of Chitosan Gel.

Gel was prepared by dissolving 2% (w/v) chitosan in 1 % (v/v) aqueous acetic acid with constant stirring at room temperature. The viscous solution prepared was filtered through a cheese cloth to remove any impurities

Antifungal activity assay of prepared Chitosan.

Anti-fungal activity of prepared chitosan was studied in vitro by a poisoned food technique (Singh et al., 2008). The Erlenmeyer flasks containing media were sterilized in an autoclave at a pressure of 15 Ib/sq inch and the temperature 121°C for 15 min. The prepared chitosan was added to sterilized media, cooled to 45°C, and shaken thoroughly. To avoid bacterial contamination we added Streptomycin to the media before pouring into Petri dishes (90 mm diameter). The final concentrations of chitosan were 125, 250, 500, 1000, 2000 and 4000 ppm. The media were then poured into a set of three petri dishes (three replicates) under aseptic conditions in a laminar flow chamber with filter (Labconco Corporation, Kansas City, Missouri 6432). After partial solidification of the media in the plate, a disc (4 mm diameter) of the fungal species was cut from 1-week-old culture with the help of a cork borer was inoculated to the center of the poured petri plates of treatments.

RESULT AND DISCUSSION

FTIR Spectra of Chitosan

The structure of the prepared chitosan was confirmed by FTIR analysis. The spectra of chitosan figure (2) showed broad band absorption around attributed to –NH and -OH stretching vibration, as well as inter and intra molecular hydrogen bonding in chitosan molecules. The band observed at 2920 and 2864 corresponds to asymmetric stretching of CH_3 and CH_2 in the prepared chitosan (Guo et al. 2005). The intensive peak around 1652 cm^{-1} corresponds to bending vibration of NH_2 which is a characteristic feature of chitosan polysaccharide and also indicates the occurrence of deacetylation (Zhang et al. 2011). The spectra also shows the peaks around 1427 &1450 cm^{-1} contributing to –CH_2COOH group, moderate peaks at 1652& 1597 cm^{-1} referred to C=O group of acetyl groups. The NH_2 stretch of amide groups & CH_2 stretch, C-CH_2, C-O and NH out-of-plane, all these peaks are at 2920, 1427, 1090 and 640 cm^{-1} respectively.

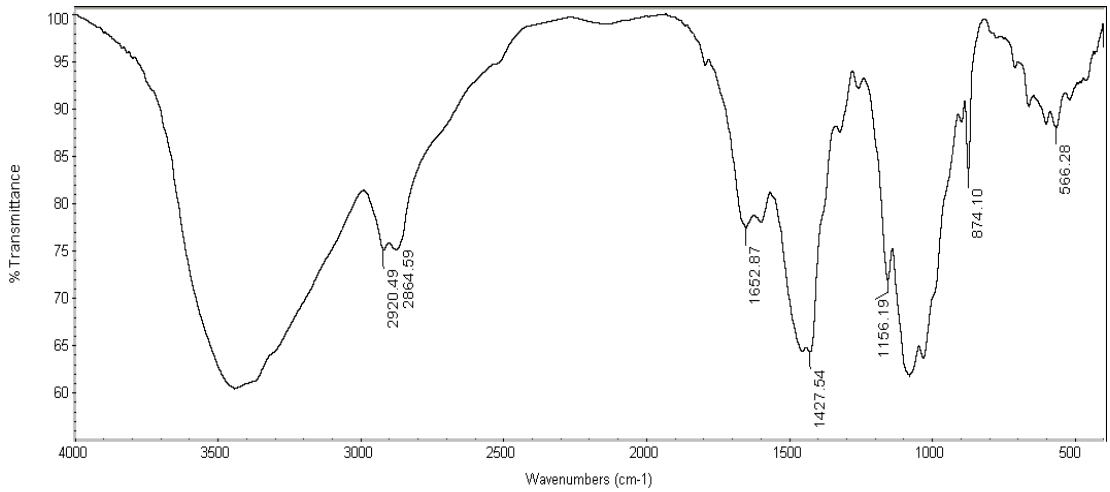


Fig 2: FTIR Spectra of prepared Chitosan.

Degree of N-deacetylation of prepared chitosan.

The degree of N-deacetylation of prepared chitosan refers to the percentage of primary amino groups in the total glycosaminoglycans after the chemical or enzymatic N-deacetylation of natural chitin. It is one of the most significant characteristics that affect the functional properties of a particular chitosan. As the N-deacetylation % increases, the acid solubility, the metal ion uptake capacity and the over all reactivity of chitosan increases. This is due to the higher reactivity of the amino groups. FTIR is one of the best tools to quantify the N-deacetylation % of chitosan from FTIR, figure 2, the N-deacetylation % of the chitosan, used in this study, was found to be 61.67 %.

Average molecular weight of prepared chitosan.

Molecular weight is also one of the significant characteristics that control the functional properties of chitosan. Viscosity is one of the simple techniques that is widely used for estimation of the molecular weights of polymers**.** The viscosity-average molecular weight (M_v) of the prepared chitosan sample was determined by using an Ostwald viscometer at 25°C in 0.3M acetic acid and 0.2M sodium acetate buffer solution and were calculated by using equation [2]. AS shown in the figure (3), the intrinsic viscositie [**η**] of the prepared chitosan CH sample, are =3.5 dl/g and the viscosity-average molecular weight (Mv) was 99,998 Da.

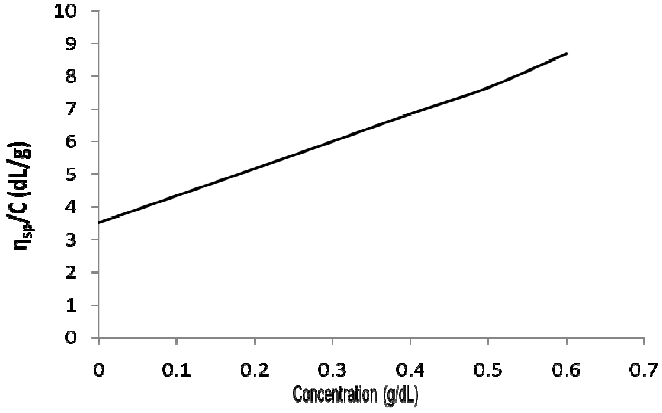


Fig 3: The reduced viscosity of chitosan vs. concentration at 25°

Degree of Swelling

Swelling is the most significant characteristic of hydrogels and it reflects the affinity of the chemical

structure of hydrogels for water and other surrounding fluids. It is evident from the results of the swelling properties represented in the Figure (4) that, they are affected by the, pH and temperature. The chitosan polymer contains more amino groups,-NH_2, which posses a high degree of deactylation, easily protonated to form more -NH_3^+ groups in acidic solution (acetic acid). The high swelling properties is due to the strong electrostatic interaction between the -NH_3^+ groups of chitosan and hydroxyl group of water. These properties decreased at pH = 4, but increased at pH=7 at 25°C and pH=7 at 37°C. These results are attributed to the increase in the mobility of the molecules with increase of temperature; this leads to increase in the porosity of the surface of the chitosan molecules and increase in its swelling properties. At pH<7, the decrease in the ratio of –OH groups leads to decrease in the swelling properties. The chitosan polymer which contains more amide groups can not be easily protonated in acidic solutions. Since the electron withdrawal by carbonyl groups makes the nitrogen of the amide groups a much poorer source of electrons than that of the amino groups, and so the electrons are less available for sharing with hydrogen ions.

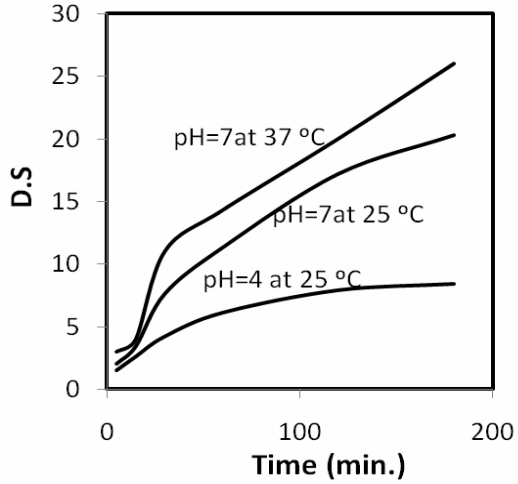


Fig 4: The degree of swelling of chitosan samples, (at different pH & temperatures) at time intervals.

X-ray diffraction (XRD) study.

The XRD pattern of chitosan prepared from shrimp shells waste at Figure (5) exhibits the weak diffraction peaks centered at diffraction angle 2θ =11.9º and sharp diffraction peaks at 2θ =20º are indicative of the high degree of crystallinity morphology of chitosan.

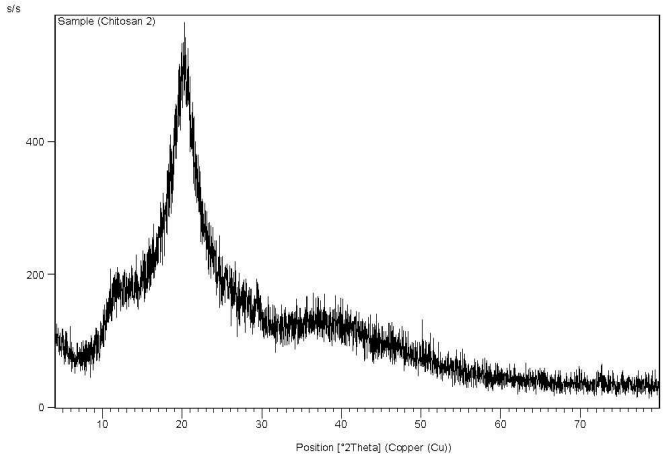


Fig 5: The XRD diffraction pattern of chitosan

Scanning electron microscopy (SEM).

Figure (6) shows the SEM (scanning electron microscopy) micrograph of prepared chitosan from shrimp shells. As it is known, SEM is one of the most widely used surface diagnostic tools. In the present study, it is visible from micrographs that the chitosan sample have smooth surface.

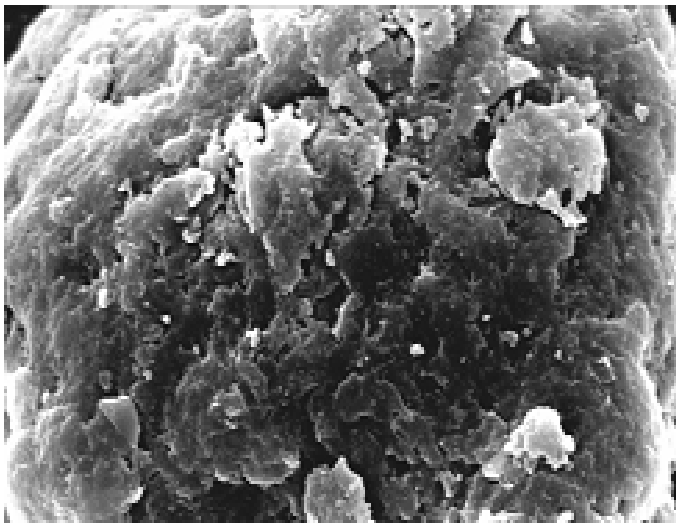


Fig 6: The SEM of chitosan film, magnification of 200 times

Thermal properties

Thermogravimetric analysis (TGA)

The previous investigations have shown that the decomposition of chitosan has 2 endothermic processes, the first one around 60°C for water evaporation, and the second one starts around 225°C and reaches a maximum 260°C. Thermal degradation of pure chitosan, as shown, can have charred residue of up to 39% at 450°C .Figure (7).

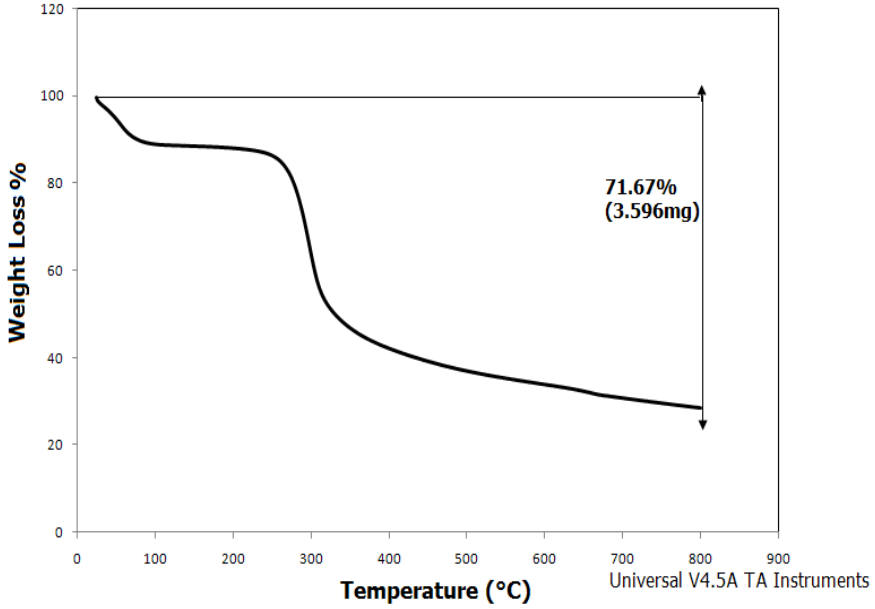


Fig 7: Thermogravimetric analysis of prepared chitosan.

Antifungal activity of the prepared chitosan.

Fungal infections have emerged as a major cause of plant morbidity. The use of synthetic chemicals as antimicrobials has greatly contributed to management of such losses, but indiscriminate application of chemicals has led to a number of ecological and health associated problems due to residual toxicity, carcinogenicity, teratogenicity, hormonal imbalance, spermatotoxicity, etc. (Kumar et al., 2007). Moreover, with the increasing incidence of strains of fungi with multiple antibiotic resistances and the persistence of fungal infections, it is of great importance to find effective treatments of infection by fungi and other microbial pathogens. Studies on novel, broad-spectrum and biodegradable antifungal agents might give crop better protection, avoiding infection by fungal pathogens.

Effect of the prepared chitosan on the linear growth of tested phytopathogenic fungi.

The result of antifungal assays of prepared chitosan is shown in Table (1)**.** We have calculated growth percentage inhibition due to treatment against control, using the following formula (Cakir et al., 2004).

$$\text{Percentage inhibition} = \left(\frac{C - T}{C}\right) x100 \qquad (4)$$

where C is the average of three replicates of hyphal extension (mm) of control, and T is the average of three replicates of hyphal extension (mm) of plates treated with tested material (chitosan). EC_{50} values were determined by the linear regression (LPd line Computer Program) of the probit of the tested fungus percentage inhibition vs. logs the concentrations (ppm) of the prepared chitosan. The EC_{50} notation used to indicate the effective concentration (ppm) that causes 50% growth inhibition. In essence, the lower the value of EC_{50} is the higher the efficacy of prepared chitosan in the test under consideration. The different values of EC_{50} for prepared chitosan tested against different fungi are compiled in Table (1) and Figures (8). The antifungal activity of chitosan prepared from shrimp shells waste at the concentrations ranged from 125 to 4000 ppm. The results showed that the percentage inhibition of mycelial growth increase with increasing concentrations of prepared chitosan for all tested strains in a dose-manner. These results were consistent with the work of Liu et al., (2006) who had demonstrated that with the increase of concentrations, the antibacterial activity of chitosan enhanced. The half inhibitory concentration of antifungal activity is expressed as EC_{50}. It was clear that *M.phaseolina*; *F.oxysporum* and *Phytophthra sp* showed strong sensitivity to chitosan. The EC_{50} values were 430.63, 566.23 and 677.22 ppm, respectively. Whereas it displayed moderate high antifungal activity against *Remularia sp*, *F.solani*, *phoma sp* and *A.solani.* The EC_{50} values were 930.93, 1054.2, 1069.22 and 1426.56 ppm, respectively. The exact mechanism of the antimicrobial action of chitosan is still unknown, but different mechanisms have been proposed. Helander, (2001) reported that the dissolve water-insoluble chitosan increased the permeability of cell membrane, and ultimately disrupted cell membranes with the release of cellular contents. Rhodes et al., (2006), proposed that water-insoluble chitosan molecules could precipitate and stack on the microbial cell surface, thereby forming an impervious layer around the cell. Such a layer can be expected to prevent the transport of essential solutes and may also destabilize the cell wall beyond repair thereby causing several leakage of cell constituents and ultimately cell death. In this work, the possible reason for the antifungal activity of prepared chitosan was supposed as follows: chitosan could bind on the microbial cell surface to form a film around the cell, so the transport of nutrient into the cell was disturbed. Also, the activity of chitosan has been explained as being based on the electrostatic interaction of the charged amino groups of chitosan with negatively charged cell wall surface of the targeted microorganisms, which can lead to the disruption of the cell wall and therefore to the death of the cell. Another possibility for antifungal activity of chitosan accounts for its chains to cross the cell membrane inhibiting the cell growing from inside (Guo et al., 2007).

Table (1). Fungicidal activity of prepared Chitosan on some phytopathogenic fungi.

Tested Fungi	% inhibition at different concentrations (ppm)						
	125	250	500	1000	2000	4000	EC_{50}
M. phaseolina	10.74	53.71	60.74	66.30	74.07	100	430.63
F oxysporum	14.82	25.55	30.37	73.33	83.33	100	566.23
Phytophthra sp	15.93	32.22	37.41	60.77	73.71	100	677.22
Remularia sp	0.0	27.41	36.33	54.07	62.92	100	930.93
F.solani	0.0	15.18	31.48	36.30	75.55	100	1054.26
phoma sp	0.0	5.18	44.07	55.18	57.41	100	1069.22
A.solani	0.0	10.37	23.71	41.85	59.63	75.20	1426.56

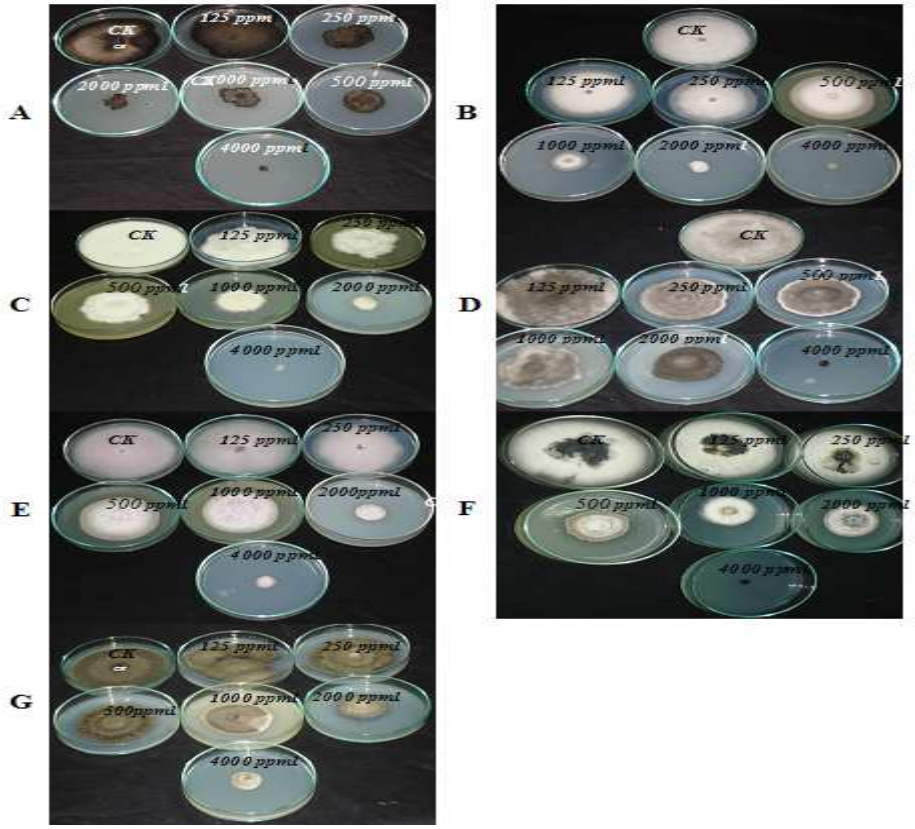


Fig 8: The antifungal effects of chitosan from shrimp shells waste against *Macrophomina. Phaseolina* (A), *Fusarium oxysporum* (B), *Phytophthra sp* (C), Remularia *sp* (D), *F.solani* (E), *phoma sp* (F) and *A.solani* (G) at different concentrations, CK represented negative control (without chitosan added).

CONCLUSION

In conclusion, the present study demonstrated that chitosan had been successfully prepared from shrimp shells waste. The preparation of chitosan from shrimp processing waste (Shells) would minimize the environmental pollutants. It was found that, chitosan is an effective antifungal agent against *F.solani*; *F.oxysporum*; *Phytophthra sp*; *macrophomina phaseolina*; *Alternaria solan*i; *phoma sp* and *Remularia sp*. The antifungal activity of prepared chitosan had relationship to its concentration and the higher concentration resulted in higher antifungal activity. Chitosan may be considered as a potential alternative to synthetic fungicides because it is a low mammalian toxicity, biodegradable and do not persist in the environment. However, further studies could be performed on the formulation of chitosan in the form of hydrogel and also compare the activity of prepared chitosan formulation with the commercial fungicides.

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REFERENCES

[1] Montesinos, E., **2003**. Development, registration and commercialization of microbial pesticides for plant protection. Int. Microbiol. 6, 245-252.
[2] Kusuma, I.W., Itoh, K., Tachibana, S., **2005**. Antifungal Activities against Plant Pathogenic Fungi of Flavonoids Isolated from Amboyna Wood, Pakistan Journal of Biological Science.8, 136-140.
[3] Bajpai, V.K., Kim, H.R., Hou, C.T., Kang, S.C., **2009**. Bioconverted products of essential fatty acids as potential antimicrobial agents, N. Biotechnol. 26, 122-130.
[4] Wilson, R.A., Talbot, N.J., **2009**. Fungal physiology-a future perspective,Microbiology. 155, 3810–3815.
[5] Gautam, A.K., Sharma, S., Avasthi, S., Bhadauria, R. **2011**. Diversity, Pathogenicity and Toxicology of A.niger: An Important Spoilage Fungi, Research Journal of Microbiology. 6, 270-280.
[6] Spadaro, D., & Gullino, M. L. (**2004**). State of the art and future prospects of the biological control of postharvest fruit diseases. International Journal of Food Microbiology, 91, 185-194.
[7] Kumar, R., Mishra, A. K., Dubey, N. K., Tripathi, Y. B., **2007**. Evaluation of Chenopodium ambrosioides oil as a potential source of antifungal, anti- aflatoxigenic and antioxidant activity. International Journal of Food Microbiology. 115, 159-164.
[8] Wilson, C. L., Solar, J. M., El Ghaouth, A., Wisniewski, M. E., **1997**. Rapid evaluation of plant extracts and essential oils for antifungal activity against Botrytis cinerea. Plant Disease. 81, 204-210.
[9] Ben-Yeohshua, S., Mercier, J., **2005**. UV irradiation, biological agents, and natural compounds for controlling postharvest decay in fresh fruits and vegetables. In: Ben-Yehoshua, S. (Ed.), Environmentally Friendly Technologies for Agricultural Produce Quality. S. Taylor & Francis, Boca Raton, pp. 266–299.
[10] Meng, X.H., Li, B.Q., Liu, J., Tian, S.P., **2008**. Physiological responses and quality attributes of table grape fruit to chitosan preharvest spray and postharvest coating during storage. Food Chem. 106, 501-508.
[11] No, H. K., Meyers, S. P., Prinyawiwatkul, W., & Xu, Z. (**2007**). Applications of chitosan for improvement of quality and shelf life of foods: a review. Journal of Food Science, 72, 87–100.

[12] Muzzarelli, R.A., **2009**. A chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. Carbohyd. Polym. 76, 167-182.
[13] Jayakumar, R., Menon, D., Manzoor, K., Nair, S.V., Tamura, H., **2010**. Biomedical applications of chitin and chitosan based nanomaterials - a short review. Carbohyd. Polym. 82, 227–232.
[14] Lin, S. B., Lin, Y. C., Chen, H. H. **2009**. Low molecular weight chitosan prepared with the aid of cellulase, lysozyme and chitinase: characterisation and antibacterial activity. Food Chemistry. 116, 47-53.
[15] Zhang, A.J., Qin, Q.L., Zhang, H., Wang, H.T., LiXuan, M.L., Wu, Y.J. **2011**.Preparation and characterization of food grade chitosan from housefly larvae. Czech J Food Sci.29 (6), 616-623.
[16] Jayakumar, R., Prabaharan, M., Sudheesh Kumar, P.T., S.V., Tamura, H., 2011. Biomaterials based on chitin and chitosan in wound dressing applications. Biotechnol Adv.29 (3): 322-37.
[17] Kumar, M. N. V. (**2000**). A review of chitin and chitosan applications. Reactive Functional Polymer, 46, 1–27.
[18] El Tahlawy, K. F., El Benday, M. A., Elhendawy, A. G., & Hudson, S. M. (**2005**). The antimicrobial activity of cotton fabrics treated with different crosslinking agents and chitosan. Carbohydrate Polymer, 60, 421- 430.
[19] Chi, F. H., & Cheng, W. P. (**2006**). Use of chitosan as coagulant to treat wastewater from milk processing plant. Journal of Polymer and the Environment, 14,411–417.
[20] US FDA (US Food and Drug Administration), **2001**. Center for Food Safety and Applied Nutrition. Office of Premarket Approval. GRAS Notices received in 2001.
[21] Atlas, M.R., **1995**. Handbook of Microbiological Media for the Examination of Food. CRC Press, Inc.
[22] Abdeen, Z. Swelling and Re-swelling Characteristics of Cross-linked Poly (vinyl alcohol) / Chitosan Hydrogel Film. Dispersion Science Technology journal, **2011**, 32(9), 1337 -1344
[23] Abdeen, Z. Preparations and Applications of Some Friendly Environmental Compounds Ph.D. Thesis, Ain-Shams University, Cairo, 2005.
[24] Moore, G.K. and Roberts, G.A.F. (**1980**) Int. J. Biol. Macromol., 2: 115.
[25] Blair, H., et al. (**1987**) J. Appl. Polymer Sci., 33: 64.
[26] Ferrus, R. and Pages, P. (**1977**) Cell. Chem. Technol., 11: 633.
[27] Singh, P., Srivastava, B., Kumar, A., Kumar, R., Dubey, N.K., Gupta, R., **2008**. Assessment of Pelargonium graveolens oil as plant-based antimicrobial and aflatoxin suppressor in food preservation. Journal of the Science of Food and Agriculture .88, 2421–2425
[28] Guo, M., Diao, P., Cai, S. **2005**. Hydrothermal growth of well aligned ZnO nanorodarrays: dependence of morphology and alignment ordering upon preparing conditions. J Solid State Chem .178(6), 1864–1873.
[29] Cakir, A., Kordali, S., Zengin, H., Izumi, S., Hirata, T., **2004**. Composition and antifungal activity of essential oils isolated from Hypericum Hyssopifolium and Hypericum heterophyllum. Flauour Fragrance. 19 (1), 62–68.
[30] Liu, N., Chen, X.G; Park, H.J., Liu, C.G; Liu, C.S., Meng, X.H., Yu, L.J. **2006**.Carbohydr.polymer.64, 60-65.
[31] Helander, I. M., **2001**. Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. International Journal of Food Microbiology .71: 235-244.
[32] Rhoades, J., Gibson, G., Formentin, K., Michael, B., and Rastall, R., **2006**. Inhibition of the adhesion of enteropathogenic *Escherichia coli* strains to HT-29 cells in culture by chito-oligosaccharides. Carbo. Polym, 64: 57-59.
[33] Guo, Z.Y., Xing, R., Liu, S., Zhong, Z., Ji, X., Wang, L., et al., **2007**.The influence of

the cationic of quaternized chitosan on antifungal activity. Int J Food Microbiol. 118(2), 214-7.