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EFFICACY OF SHRIMP SHELL SYNTHESIZED CHITOSAN AGAINST POST-HARVEST SPOILAGE FUNGI OF *Sesamum indicum*

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ABSTRACT
Contamination of Agricultural commodities by moulds poses significant challenges due to conducive climatic conditions. Mycotoxins produced by moulds like *Aspergillus*, *Penicillium*, and *Fusarium* present health risks and economic losses. Traditional control measures such as the use of fungicides have drawbacks, necessitating the exploration of eco-friendly alternatives. One such alternative is the use of Chitosan, a biopolymer derived from chitin found in crustacean exoskeletons. This study evaluated the efficacy of chitosan synthesized from shrimp shells in inhibiting the growth of *Aspergillus niger*, isolated from stored Sesame seeds. Synthesized Chitosan was characterized using FTIR spectroscopy, and UV-visible spectroscopy, and the degree of deacetylation was determined. FTIR analysis showed characteristic absorption peaks ranging from 3911.77 to 794.70 cm^{-1}, with higher wave numbers corresponding to stretching vibrations of the C-H, O-H, and N-H bond functional groups. UV-visible spectroscopy displayed an absorbance peak at 232 nm with an absorbance value of 2.15. The degree of deacetylation of synthesized chitosan was determined to be 98.99%. In vitro, experiments showed a concentration-dependent inhibition of *A. niger* growth by shrimp shell-synthesized chitosan. The highest level of inhibition was seen at day 3 with 0.75 mg/ml (50%) and 1.0 mg/ml (52.1%), indicating that the efficacy of shrimp shell synthesized chitosan was concentration dependent. Results suggest chitosan is a potential anti-fungal agent for food preservation against mould contaminants, mitigates post-harvest losses and ensures food safety.

Keywords: Moulds, chitosan, inhibitory, oil seeds

INTRODUCTION
In tropical countries like Nigeria, mould contamination in agricultural commodities such as oil seeds is complicated to avoid, as climatic conditions such as high humidity, rainfall, and temperatures support their growth (Alburquenque *et al*., 2010). One such oil seed is Sesame (*Sesamum indicum*) widely grown in Nigeria, which ranks fifth, on the global list of highest-producing nations. Specifically, contributed 450,000 t to the global production (FAOSTAT, 2019a). One major challenge to this economically important crop is contamination by food-borne moulds which are hazardous to human health because of their ability to produce toxic metabolites known as mycotoxins (Correa & Abreu, 2020). Common moulds that often contaminate food products are the *Aspergillus*, *Penicillium* and *Fusarium* species this is due to their ubiquitous nature (Zachetti *et al*., 2019; Binder *et al*., 2007). Considering the high impact of mould contamination on human and animal health, and the economic losses related to the contamination of food and feed stuffs. With mycotoxins, it is important to develop strategies to prevent, inactivate, or reduce their presence in food produce and products. Strategies such as the use of agronomical practices, resistant cultivars, and fungicides have been adopted (Zachetti *et al*., 2019).

However, synthetic fungicides are reported to cause problems such as chemical residues in food, alteration of the nutritional composition of foodstuff, pollution and the development of resistant fungi (Zachetti *et al*., 2019). According to Liang *et al*. (2018), synthetic fungicides may also act as stress factors resulting in the stimulation of mycotoxin production. Therefore, the need for alternative control in reducing the growth of these moulds in food is essential. These alternatives include the use of safe biological material obtained from natural sources to replace synthetic fungicides in reducing the growth of these moulds in food (El-araby *et al.*, 2022).

Chitosan biomaterial is one of the alternate ways to overcome the problem of the presence of *Aspergillus* contaminant in food (Younes & Rinaudo, 2015; Jayakuma *et al.*, 2017). It is a promising eco-friendly alternative since it can be used to produce biodegradable fungicides (Convey *et al*., 2017; Zachetti *et al*., 2019). The polysaccharide is obtained from the deacetylation of chitin and is the second most abundant natural biopolymer found in nature after cellulose (Samiyah *et al.*, 2021; Ulaganathan *et al.*, 2017). It is edible, accessible, non-toxic, and biocompatible and can be extracted from various sources, particularly exoskeletons of crustaceans (Du *et al*., 2009). One of the most attractive features of chitosan is its antibacterial, antiviral and antifungal activities

(Jayakuma *et al.*, 2017; Muzzarelli, *et al*., 2001). Cazón and Vázquez (2019) and Zhang *et al*. (2011) opined that the utilization of chitosan as a food preservative in agricultural protection stimulates the defence of different crops. Hence this study seeks to assess the efficacy of chitosan to inhibit the growth of *Aspergillus niger* a spoilage mould of Sesame an economically significant oil seed.

MATERIALS AND METHODS

Collection of materials

Shrimp shells were obtained from sea shores in Warri, Delta State, Nigeria. *Aspergillus niger* previously isolated from stored sesame seeds was obtained from the microbial culture bank of the Nigerian Stored Products Research Institute (NSPRI), Ilorin, Kwara State, Nigeria. The growth medium Potato-Dextrose Agar (PDA) was also sourced from Bristol Scientific, Lagos, Nigeria.

Preparation of potato dextrose agar (PDA)

Thirty-nine (39) grams of PDA was suspended in 1000 ml distilled water and homogenized using a magnetic stirrer to dissolve the powder completely. The medium was sterilized by autoclaving at 121^{o}C for 15 minutes (Manufacturers guide)

Synthesis of chitosan from shrimp shell

Shrimp shells were prepared by washing, drying, grinding and sieving. Chitin was isolated from shrimp shells by chemical processes viz. Deproteinization, demineralization, and decolourization (Pokhrel *et al*., 2016). For Deproteinization (DP), shrimp shell powder was treated with 150 g/l of NaOH solution in a 1:10 weight/volume ratio. The mixture was heated to 60^{o}C for 3 h in a reaction flask. The obtained deproteinized powder was washed with distilled water until neutrality was reached and dried in an oven at 40^{o}C. Deproteinized (D) shrimp shell powder was subject to a demineralization process by treating the shell powder with 1M citric acid in a 1:15 weight/volume ratio. The reaction was carried out for 2 h at 37^{o}C and obtained solid was washed until neutrality of the resultant solid was chitin, which was then decolourized by 2% sodium hypochlorite solution (600 mL) in a 1:20 ratio of chitin and NaCl. The mixture was stirred for 30 minutes at 45°C. Obtained chitin was washed until neutrality was reached. Chitin deacetylation was carried out by treating with sodium hydroxide (50% solution) in a 1:10 weight/volume ratio. The reaction was carried out at 15 psi/121^{o}C. The sample was filtered off, washed with distilled water to neutral pH and dried in an oven at 60^{o}C for 24 h. (Pokhrel *et al*., 2016).

Characterization of synthesized chitosan

The degree of deacetylation of synthesized chitosan was determined by the infrared spectroscopy method and UV spectrophotometer (UV-visible 19002PC) (Yusuf *et al*., 2023). The surface plasmon resonance (SPR) band was measured by diluting a small aliquot of the sample in 1 ml of distilled water in a cuvette. The spectra between 200-800 nm ranges were scanned to find the absorbance peak of synthesized chitosan.

Fourier transform infrared (FTIR) spectroscopy of synthesized chitosan

The extracted chitosan samples were analyzed by Fourier transform infrared (FTIR) spectroscopy to determine the presence of the characteristic IR bands. Dried powdered samples were mixed with KBr and then pressed to create a homogenous sample/KBr disk. The chitosan sample's infrared spectra were measured over the frequency range of 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1} using a Bruker Vertex 70 spectrometer.

Characterization of chitosan synthesised from shrimp shell (CSSS)

UV-visible spectra scanning of Chitosan synthesis from shrimp shell

Ultra Violet-visible spectra were recorded using a UV-visible 19002PC spectrophotometer (manufactured in China) for confirmation of chitosan synthesis.

Degree of deacetylation (DD) determination

FTIR spectra of synthesized chitosan obtained in a frequency range of 400–4,000 cm^{-1} were used in the calculation of the degree of deacetylation (DD) of the extracted chitosan (El-araby *et al*., 2022)

Inhibition of mycelial radial growth of fungal isolates

The in vitro antifungal activity of chitosan synthesized from Shrimp Shells was determined using the food poisoning method. A disc (6 mm diameter) each was taken from the pure cultures of the selected fungal isolate (*A. niger*) and inoculated at the centre of each petri dish containing PDA and chitosan solutions at 0.25, 0.50, 0.75 and 1.0% concentration. A Petri dish containing PDA with sterile distilled water and glacial acetic acid was used as a control. The plates were in triplicates and incubated at room temperature (28+2°C). Daily radial growth was measured for 4 days. The percentage inhibition of radial mycelial growth (PIRG) was calculated using the formula described by Yusuf *et al*. (2023).

$$\%\text{PIRG} = \frac{R1 - R2}{R1} \times 100$$

RESULT AND DISCUSSION

Chitin obtained from crustaceans is found in a complex matrix with proteins, minerals, and pigments (El-araby *et al*., 2022). The extraction protocol for extraction of chitin from crustacean exoskeletons includes deproteinization, mineralization and decolourization which was followed in this study (Srinivasan *et al*., 2018; Convey *et al*., 2017). In this study, chitosan was obtained based on alkaline chitin deacetylation. In this study citric acid an organic acid was used in the demineralization process, El-araby *et al*. (2022) opined that the use of an organic acid is more environmentally friendly as it is significantly, less expensive, and safe for the environment.

UV-visible spectra scanning of Chitosan synthesis from shrimp shell

Figure 1 shows the graph of the UV spectrophotometer of chitosan (UV-vis) synthesized from shrimp shells at a wavelength of 232 nm and an absorbance peak of 2.15.

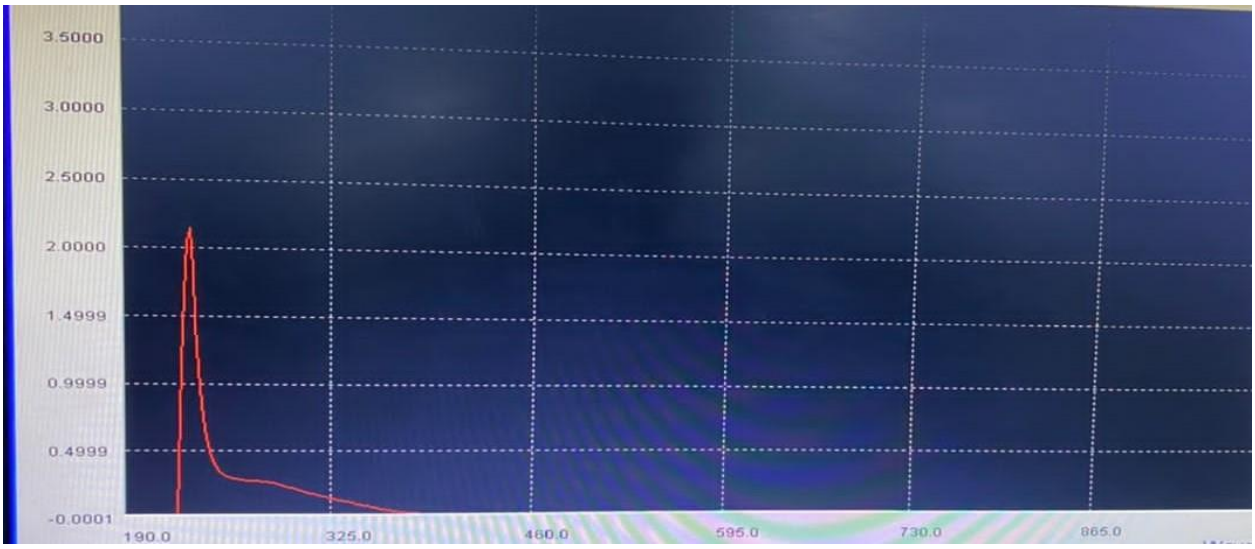


Figure 1: Graph of UV spectrophotometer of chitosan synthesis from shrimp shells

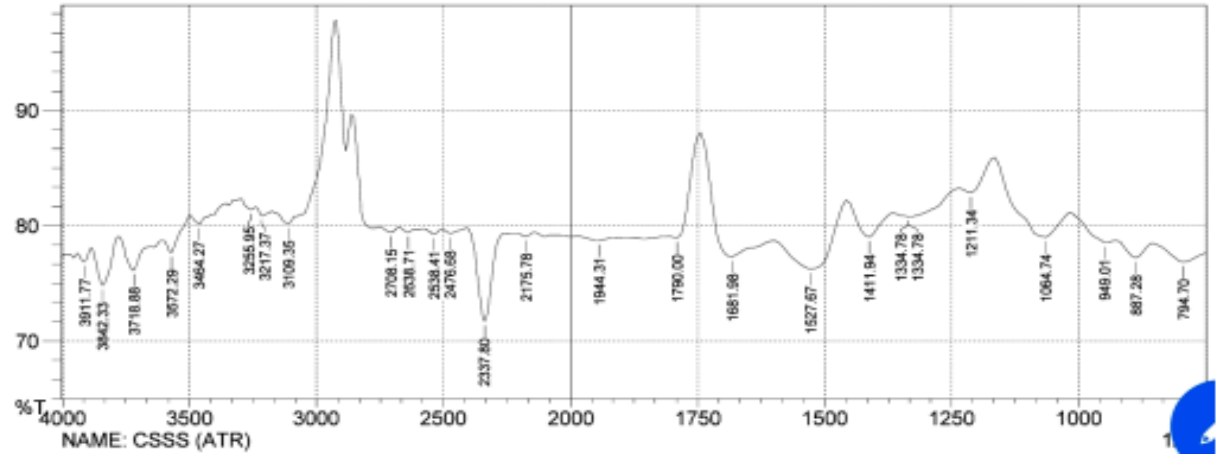


Figure 2: FTIR peaks of chitosan synthesized from shrimp shells

The final chitosan product was characterized by Fourier transform infrared (FTIR) spectroscopy to identify the functional groups in the shrimp shells synthesized chitosan. The result of the FTIR analysis (Figure 3) showed absorption peaks ranging from 3911.77 $cm^{-1,}$ 3842.33, 3718.88, 3572.27, 3464.24, 3255.95, 3217.37,3109.35, 2708.15, 2638.71, 2538.41,2476.68, 2175.78, 1944.31, 1790.00,1681.98, 1527.67, 1411.94, 1334.78,1211.34, 1064.74, 949.01, 887.28 to 794.70 cm^{-1} (Figure 2). Higher wave numbers (closer to 4000 cm^{-1}) typically correspond to stretching vibrations of the C-H, O-H, and N-H bond functional groups. While wave numbers in the mid-range (1500-1800 cm^{-1}) represent C-H bending vibrations and those below 1500 cm^{-1} typically correspond to skeletal vibrations of larger functional groups. Kumari *et al.* (2017) also observed similar FTIR bands for chitosan synthesized using shrimp shells.

Degree of deacetylation

The degree of Deacetylation DD) of extracted chitosan was calculated using the FTIR spectra. In FTIR spectra, different absorption bands represent different functional groups present in chitosan (El-araby *et al.*, 2022). In this study, deacetylation degree of chitosan synthesized using shrimp shell was 98.99%. The degree of deacetylation determines the physical, chemical, and biological properties of this chitosan (Yusuf *et al*., 2023; El-araby *et al*., 2022).

$$\frac{A_{1429}}{A_{3444}} \quad 1.15$$

Where Area of peak of 1790.00=78.944T and Area of peak of 3109=80.022T

$$A3109 = \frac{-\log T}{100} = \frac{-\log 80.022}{100} = 0.0967;$$

$$A1790 = -\frac{logT}{100} = \frac{-log78.944}{100} = 0.1026$$

$$A = 100 - \frac{0.0967}{0.1026} \times 1.15 = 98.9\%$$

Identification of target fungi

Table 1 shows a photomicrograph of *Aspergillus niger* isolated from stored S*esame indicum* got from NSPRIs microbial culture bank. There was the characteristic black colouration of *Aspergillus niger* colonies on Petri plates. The colonies were dense and cottony, with a distinct texture.

Table 1: Photomicrograph of *Aspergillus niger*

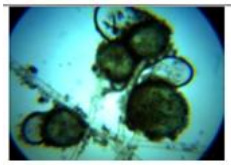
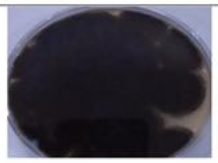
Photomicrograph	Growth on Plate	Probable fungi
		*Aspergillus niger*

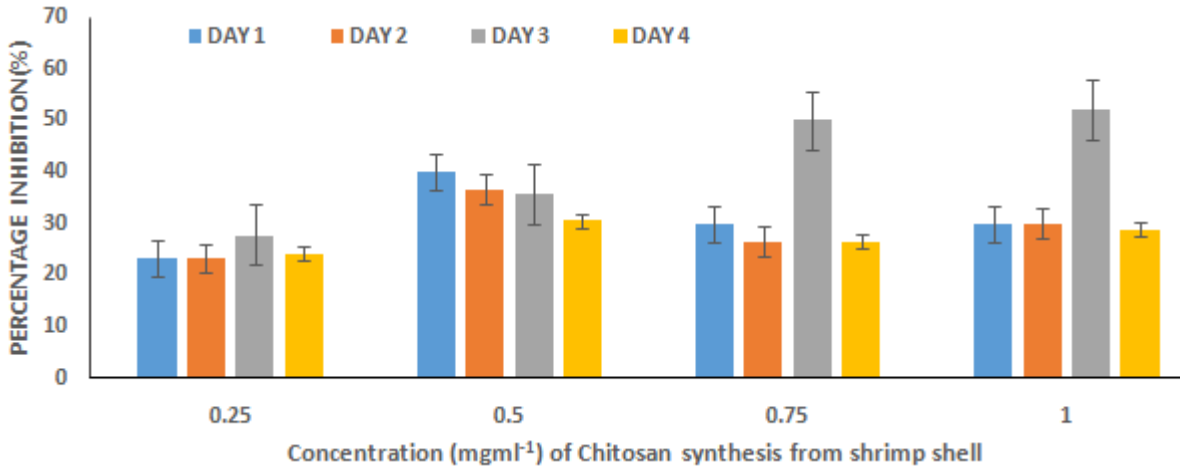
Table 2: Mean mycelia radial growth of *A. niger* in Chitosan synthesis from shrimp shell treated plates

Days of Incubation	Mean mycelial growth of *A. niger* in different concentrations (mg ml-1) of chitosan synthesis from shrimp shell				
	Control	0.25	0.50	0.75	1.0
1	1.50±.00^{b}	1.15±.15^{a}	.90±.10^{a}	1.05±.15^{a}	1.05±.15^{a}
2	1.50±.00^{b}	1.50±.15^{b}	.95±.15^{a}	1.10±.10^{a}	1.05±.15^{a}
3	4.50±.00^{b}	3.25±.25^{b}	2.90±1.0^{a}	2.25±.25^{a}	2.40±.40^{a}
4	8.55±.05^{d}	6.54±.05^{c}	5.90±.10^{b}	6.25±.55^{c}	6.05±1.25^{c}

Figures having common superscript alphabet along rows are not significantly different at P≤0.05

Mean mycelia radial growth of *A. niger* in chitosan synthesis from shrimp shell

The result of the mean mycelia growth of *A. niger* through the in vitro test at different concentrations of chitosan is shown in Table 2. Results show that on days 1 and 2 of incubation, there was no significant difference in mycelia inhibition except for the control sample (1.50 ± .00) while on day 3, the concentration of 0.50, 0.75 and 1.0 mg/ml had the least mean mycelia radial growth (2.90±1.0, 2.25±.25, 2.40 ±.40) among the treatment concentrations respectively. There was a significant difference in growth inhibition at 0.25 mg/ml and control treatments at day 4 (Table 2). There was growth in both chitosan treatments and control, however, there was a significant difference (p≤0.05) in growths across treatments with the highest mycelia growth occurring in the control samples. There were fluctuations in mycelia inhibition on all the days at concentrations of 0.75 mgml. There was a decrease in mycelia growth on days 2, 3, and 4 with the highest mycelia growth occurring on the fourth day of incubation at 6.05 ± 1.25 at a concentration of 1.0 mg/ml. It was observed that the inhibitory activity of chitosan synthesized from shrimp shell on mycelia growth of *Aspergillus niger* was not entirely concentration dependent as there were variations in the inhibitory activity of the synthesized chitosan even at higher concentrations across the different days of exposures. These results are not totally in agreement with those of El-Araby*et al*. (2022), Resmila and Rinto (2017) who opined that the higher the concentrations the lower the mycelia radial growth.



Figures having a common superscript alphabet along rows are not significantly different at P≤0.05

Figure 3: Percentage inhibition of *A. niger* in different concentrations of chitosan synthesis from shrimp shell (CSSS)

Figure 3, below shows percentage inhibition of *A. niger* through food poisoning technique. On day 1, 2, 3, 4 inhibitory activity of shrimp shell synthesized chitosan ranged from 23.3-30%, 23.3-36.6%, 27.8-52.1%, and 24.1-28.88%, respectively. The highest level of inhibition was seen at day 3 with 0.75 mg/ml (50%) and 1.0 mg/ml (52.1%), meaning the higher the concentration of shrimp shell synthesized chitosan the higher the inhibition. Percentage inhibition of *A. niger* in different concentrations of the chitosan synthesized from shrimp shell showed highest percentage inhibition with increasing concentration. In this study, growth of *Aspergillus niger* decreased significantly where chitosan concentration increased. El-araby, *et al*. (2022), Dewi and Nur (2017) reported the inhibitory effect of chitosan on mycelial growth of *Aspergillus*

niger and affirmed that the higher the percentage of inhibition the corresponding increase in inhibition. This they attributed to the (positively charged) chitosan which can bind to microorganisms with a negative charged cell surface, resulting in leakage of intracellular materials of the cell membrane (El-araby *et al*., 2022; Meng *et al*., 2020).

CONCLUSION

Based on this study, it can be concluded that chitosan has antifungal activity that can inhibit the growth of *A. niger*. A chitosan concentration of 0.75% inhibited 50% of tested *A. niger*, but a 1.0% chitosan concentration had the highest level of inhibition of 50%. The most effective concentration in preventing the growth of moulded mycelia colony was achieved with 0.2 concentration. Hence, Chitosan is an effective biomaterial for the inhibition of *Aspergillus species* and to reduce post-harvest loss of agricultural produce to fungal attack

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