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EVALUATION OF FUNGICIDAL EFFICACY OF FREE AND NANO-ENCAPSULATED CHITOSAN AGAINST *Aspergillus flavus* ISOLATED FROM RICE (*Oryza sativa*) SEED

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ABSTRACT

Grains are exposed to contamination by mycotoxins, both in the field and during storage. Aflatoxins produced by *Aspergillus* species are the most harmful and carcinogenic mycotoxins in grains. Synthetic fungicides are widely used for the control of mycotoxigenic fungi in grains. However, rising public awareness about the toxicological effects of fungicides on human health necessitates the development of non-toxic bio-fungicides. In this connection, reports have shown that chitosan synthesized from shell waste has the potential to serve as an alternative fungicide. Therefore, this study investigated the fungicidal efficacy of free and nano-encapsulated chitosan against aflatoxigenic fungus (*Aspergillus flavus*). High molecular weight chitosan was purchased, and the *in-vitro* antifungal efficacy of chitosan against *A. flavus* was tested using the food poisoning method. Nano-encapsulated chitosan was synthesized using the ionic gelation method, the particle size was determined, and *in-vitro* inhibition against *A. flavus* was investigated. The results revealed that nano-encapsulated chitosan with particle sizes of 525.4 nm, 468.3 nm, and 711.7 nm were obtained. *In-vitro* mycelial *A. flavus* growth inhibition of 100% was recorded at 1.5% and 2.0% of free chitosan, while at 0.5% and 1.0% of free chitosan, 45% and 75% inhibition were observed, respectively. Nano-encapsulated chitosan with particle size (nm) of 525.4, 468.3, and 711.7 at 0.50% recorded 61%, 84% and 87% inhibition, respectively. However, at 0.25%, the rate of inhibition was 64%, 78% and 67%, respectively. This study showed that free chitosan and nano-encapsulated chitosan are potential antifungal agents for the control of *A. flavus*.

Keywords: Fungi, Fungicide. Chitosan, Inhibition, Mycotoxin, Nano-Encapsulated.

INTRODUCTION

According to FAO estimates, more than 25% of global food crops are contaminated by mycotoxins (Eskalo *et al.,* 2019). The major mycotoxin contaminants in crops from field to storage are aflatoxins (AFTs), ochratoxins, and deoxynivalenol (DON), which are primarily produced by *Aspergillus* species, *Penicillium* species, and *Fusarium* species, respectively (Ok *et al.,* 2014; Frisvad *et al.,* 2019). Among these, the AFTs produced by *Aspergillus* species are the most harmful and carcinogenic toxins in grains and can provoke tumour formation or even rapid death (Ding *et al.,* 2015). The substantial climatic differences and geographic locations could result in varied compositions and distributions of toxigenic fungi. For example, high temperatures and high relative humidity conditions in tropical and subtropical regions favour the growth of *A. flavus* and some other fungal species (Pratiwi *et al.,* 2015).

The production of shell wastes from crabs by the seafood industry contributes to environmental and health hazards (Divya *et al.,* 2014). However, it is worth mentioning that fishery by-products have economic value for chitin and chitosan production (Ahing and Wid, 2016). Thus, conversion of shell waste to commercial products such as chitosan could be considered an alternative approach to shell waste management (Divya *et al.,* 2014). According to the Biopesticides manual, chitosan is described as a 'crustacean-derived plant defence booster with a chemical formulae of polysaccharide 2-Amino-2-deoxy-beta-D-glucosasmine (Keith *et al.,* 2019). It is also a natural, biodegradable polymer with various applications in agriculture, the food industry, biotechnology, medicine, and cosmetology (El-Hadrami *et al.*, 2010). Another attribute of this

natural compound is the fungistatic or fungicidal properties against pathogens of various crops (Madushani *et al.,* 2012). Nano-encapsulated particles are particulate dispersions or solid particles with a size ranging from 1 to 1000 nm. Techniques such as emulsion, ionic gelation, reverse micellar, and self-assembling methods are utilized to prepare chitosan nanoparticles (Zhao *et al.,* 2011). Applications of chitosan nanoparticles in agriculture are encouraged because of their higher mobility and surface area for efficient delivery of agrochemicals and micronutrients. Its applications as antifungal and antibacterial agents, hormone delivery, enhancement in respiration rate, increment in seed germination rate, and enhancement in growth and yield have been reported (Kashyap *et al.,* 2015). The study aimed to evaluate the *in vitro* antifungal efficacy of free chitosan, synthesize and characterize nano-encapsulated chitosan, and evaluate its inhibitory effect on the growth of *Aspergillus flavus*.

MATERIALS AND METHODS

Collection of materials

High molecular weight chitosan (HMWC) (MW 760 kDa; ⩾ 85%)) was obtained from Chitin-Chitosan BioChemika and Sigma- Aldrich Company USA. The growth medium (Potato-Dextrose Agar (PDA) and Sodium Tripolyphosphate (TPP) were also sourced from Hi-media Company, India.

Identification of *Aspergillus flavus*

Previously isolated *A. flavus* from stored rice seeds was identified using the World Mycological Monograph (Adebola and Amadi, 2012). Subcultures were obtained from old stock cultures. Identification of isolated fungi was performed using their macroscopic appearance and microscopic features. The microscopic examination was conducted by using a sterile inoculating wire loop to put one drop of distilled water on a slide. Then, the spores and fragments of mycelium were transferred from the new subculture plate onto the slide. The mycelium was teased, two drops of cotton blue in lactophenol were added, and a cover slip was placed on it. The slide was viewed under lower magnification of the stereo microscope (Olympus, Japan) until the conidiophores and conidiospores were identified.

Preparation of chitosan solution

Chitosan solutions were prepared by weighing 0.5, 1.0, 1.5, and 2.0 g of chitosan and dissolved in 100 mL sterile water containing 0.5 mL (v/v) of glacial acetic acid. The mixture was placed on 85-1 overhead Magnetic stirrer (Jiangsu Jinyi Instrument Technology Company Limited, China) at 600 rpm for 6 h. The pH of the solution was adjusted to 5.6 by adding 1 N HCl, depending on the pH reading, using a digital pH meter model PHS-25 (Jiangsu Zhengji Instruments Co. Ltd, China) (Madushani *et al.,* 2012).

Synthesis of nano-encapsulated chitosan

Nano-encapsulated chitosan was synthesized using ionic gelation methods (Kashyap *et al.,* 2015). High molecular weight chitosan (HMWC) solutions (0.1 M) were prepared by dissolving 16 g of chitosan in 1000 mL of 2% glacial acetic acid with stirring using 85-1 Magnetic stirrer (Jiangsu Jinyi Instrument Technology Company Limited, China) at 600 rpm for 6 h at a temperature of 60°C. Thirty-seven grams (37 g) of Sodium Tripolyphosphate (Tpp) was also dissolved in 1000 mL of double distilled water to prepare a 0.1 M solution. Nano-encapsulated chitosan of different degrees of cross-linking and diameters was prepared by adding the chitosan solution dropwise to Tpp solution in ratios (1:1, 1:3, and 1:5 v/v). The resultant Nano-encapsulated chitosan was filtered, washed several times with double distilled water, and oven-dried at 60°C (Zahid *et al.,* 2015).

Characterization of nano-encapsulated chitosan

Ultra Violet-visible spectra were recorded using a UV-visible HZ 1902 Double Beam spectrophotometer (Huazheng Electric Manufacturing Co. Limited, Hebei, China) to confirm nanoparticle formation. Dynamic light scattering (DLS) was used for the measurement of average particle size and polydispersity index (PDI) on a high-performance particle Zetasizer HPPS-5001 (Malvern, UK). Each sample was analyzed in triplicates at 25°C at a scattering angle of 90°C. Sterile water was used as a reference for dispersing the medium. The results were recorded as the average particle size obtained from the analysis of three batches in triplicate (Zulfajiri *et al.,* 2020).

Inhibition of mycelial radial growth of fungal isolates

The *in-vitro* antifungal activities of chitosan (HMWC) and nano-encapsulated chitosan were determined using the food poisoning method. A disc (6 mm diameter) each was taken from the pure cultures of a three-day-old *A. flavus* and inoculated at the centre of each Petri dish containing PDA and chitosan solutions at 0.5%, 1.0%, 1.5% and 2.0% and nano-encapsulated chitosan at 0.25% and 0.50%. A Petri dish containing PDA with sterile distilled water and glacial acetic acid was used as a control. The experiment was a Completely Randomized Design (CRD). The plates were replicated three times and incubated at room temperature (28±2 °C) (Aremu *et al.,* 2023). Daily radial growth was measured for 5 days (Zahid *et al.,* 2015). The percentage inhibition of mycelial radial growth (PIRG) was calculated at the end of the fifth incubation day using the formula described by Al-Hater *et al.* (2010).

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

Where R1 = mycelial growth in control plates, R2 = mycelial growth in treated plates. Data generated were subjected to analysis of variance (ANOVA) using Statistical Tools for Agricultural Research (STAR) and mean separation using LSD at a 5% (0.05) level of probability.

RESULTS

Morphological and microscopic identification of *Aspergillus flavus*

Aspergillus flavus has a velvety texture with a light green to yellowish colony bound by a white to cream edge on a potato dextrose agar plate (Plate 1a). The microscopic characteristic showed non-septate hyphae and unbranched conidiophores with scanty sterigmata. Conidia with varying sizes that are slightly roughened were observed (Plate 1b).

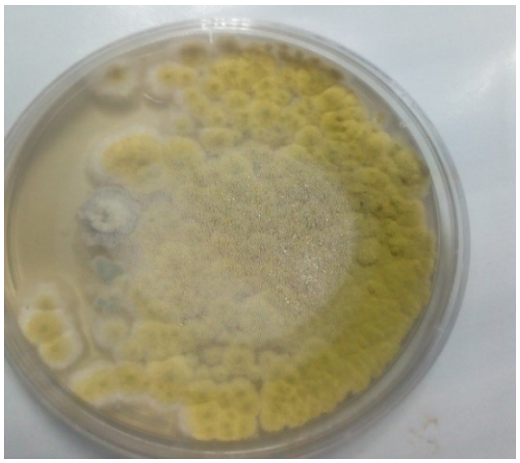


Plate 1a: *Aspergillus flavus* on PDA

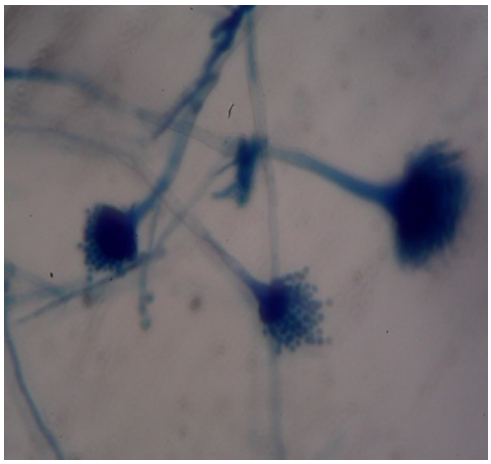


Plate 1b: Photomicrograph of *A. flavus* (x 100)

Plate 1: Macroscopic appearance and photomicrograph of *Aspergillus flavus* isolate

Nano-encapsulated chitosan (N-ECs) synthesis and characterization

The size of CNPs at the selected ratio of chitosan (Cs) and sodium tripolyphosphate (TPP) ranged from 468.3 nm to 711.7 nm. It was also observed that 1:5 Cs/Tpp produced a higher nanoparticle size of 711.7 nm while 1:3 Cs/Tpp produced a smaller particle size of 468.3 nm. (Table 1). The poly disparity index ranged from 0.433 to 0.631.

Table 1: Characterization of synthesized Nano-encapsulated chitosan.

Chitosan	Cs: Tpp	UV Spectroscopy Wavelength (nm)	Absorbance peak (A)	Nanoparticle size Synthesis (nm)	Poly disparity index (PdI)
HMWC	1:1	231	2.652	525.4	0.483
HMWC	1:3	233	0.425	468.3	0.433
HMWC	1:5	233	1.420	711.7	0.631

The results showed that *in-vitro* mycelia radial growth of *A. flavus* was observed in the chitosan treatment and control groups (Table 2). However, there was a significant difference ($P<0.05$) between the treatment and control groups. The highest mycelia radial growth of 8.00 cm was recorded with the control and significantly different from the mycelia radial growth of 1.80 cm in 0.5% chitosan treatment. However, there was no growth at 1.5 and 2.0% chitosan-treated plates.

Table 2: Mycelia radial growth of *A. flavus* in different concentration of chitosan.

Days of Incubation	Concentrations of chitosan (%)				
	0	0.5	1.0	1.5	2.0
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
1	2.50^{b} ± .06	0.00^{a} ± .00	0.00^{a} ± .00	0.00^{a}± .00	0.00^{a} ± .00
2	5.00^{b} ± .06	1.17^{a} ± .73	0.33^{a} ± .33	0.00^{a} ± .00	0.00^{a} ± .00
3	6.50^{b}± .06	1.33^{a} ± .73	0.50^{a} ± .50	0.00^{a} ± .00	0.00^{a} ± .00
4	7.00^{b}± .06	1.50^{a} ± .87	0.67^{a} ± .67	0.00^{a} ± .00	0.00^{a} ± .00
5	8.00^{b}± .06	1.80^{a} ± .04	0.67^{a}± .67	0.00^{a} ± .00	0.00^{a} ± .00

Note: Values in the same row and column not sharing the same superscript are significantly different at $P< 0.05$ in the two-sided test of equality.

The result of the *A. flavus* mycelia radial growth in nano-encapsulated chitosan treatment (Table 2) showed that the higher the concentration of nano-encapsulated chitosan, the lower the mycelia radial growth, thus the higher the inhibition. The highest mycelia radial growth of 8.00cm was recorded in control (0%), which was significantly different from the mycelia radial growth of 3.10cm in 0.25% concentration of (1:1) HMWC:Tpp nano-encapsulated chitosan with particle size 524.4nm. The lowest mycelial radial growth of 1.07cm was recorded in 0.50% concentration of (1:5) HMWC:TPP nano-encapsulated chitosan with particle size 711nm.

Table 3: Mycelia radial growth of *A. flavus* in different concentration of nano-encapsulated chitosan.

Days of Incubation	Concentration of Nano-encapsulated chitosan (%)						
	1:1(525.4nm)			1:3(468.3nm)		1:5(711.7nm)	
	0%	0.25%	0.50%	0.25%	0.50%	0.25%	0.50%
1	3.00^{d}±.06	1.10^{a}±.12	$0.93^{a,c}$±.03	$1.^{00a,c}$± .00	0.00^{b}±.00	0.80^{c}± .06	0.00^{b}± .00
2	6.00^{d}±.06	1.80^{a}±.12	1.80^{a}± .12	1.50^{a}± .06	0.80^{b}±.06	1.47^{a}± .07	1.00^{b}± .00
3	7.50^{i}±.06	2.53^{a}±.09	2.00^{b}± .06	2.07^{b}± .07	0.77^{c}±.07	1.57^{d}± .09	$1.00^{c,e}$±.00
4	8.00^{i}±.06	2.90^{a}±.06	$2.37^{b,d}$±.09	$2.70^{a,b}$± .12	1.00^{c}±.12	$2.00^{d,e}$± .12	1.00^{c}± .06
5	8.00^{i}±.06	3.10^{a}±.12	$2.87^{a,e}$± 09	$2.73^{a,c,g}$±37	1.13^{b}±.13	$2.07^{c,f,g,h}$±.07	1.07^{b}± .07

Note: Values in the same row and column not sharing the same superscript are significantly different at $P< 0.05$ in the two-sided test of equality

The results of the chitosan percentage inhibition of mycelial radial growth of *A. flavus* (Figure 1) show that 100% inhibition was recorded in 1.5% and 2.0% concentration of chitosan, while 47.5% inhibition was recorded in 0.50% chitosan concentration which was significantly different ($P<0.05$) from 1.5% concentration. Nano-encapsulated chitosan's highest percentage inhibition of 87.18% was recorded in 0.50% concentration of 1:5 Cs/Tpp with 711.7 nm size particle, and the lowest (61.53%) was 0.50% concentration of 1:1Cs/Tpp with 525.4 nm particle size at the end of day 5 of incubation.

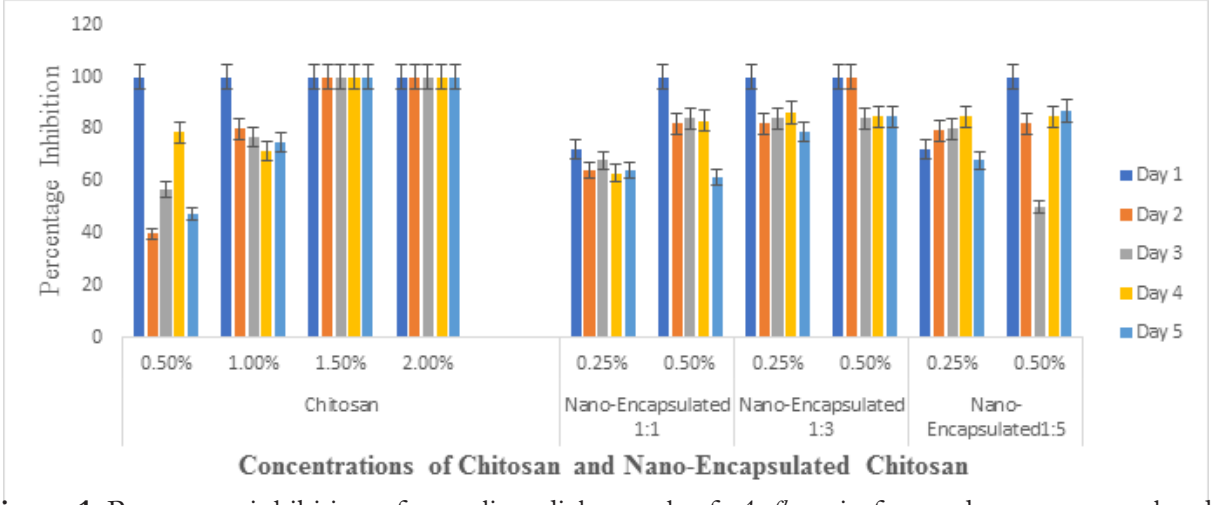


Figure 1: Percentage inhibition of mycelia radial growth of *A. flavus* in free and nano-encapsulated chitosan**.** Percentage with the same error bar are not significantly different at the 5% level of significance in Statistical Tools for Agricultural Research (STAR) Package

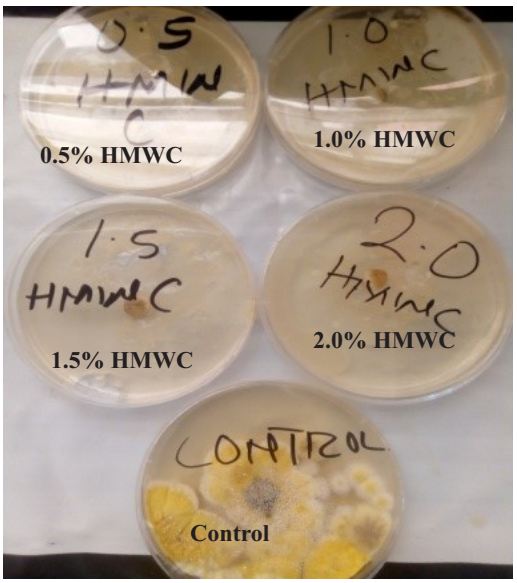


Plate 2: Growth inhibition of *Aspergillus flavus* in different concentration of high molecular weight chitosan (HMWC) in its free form.

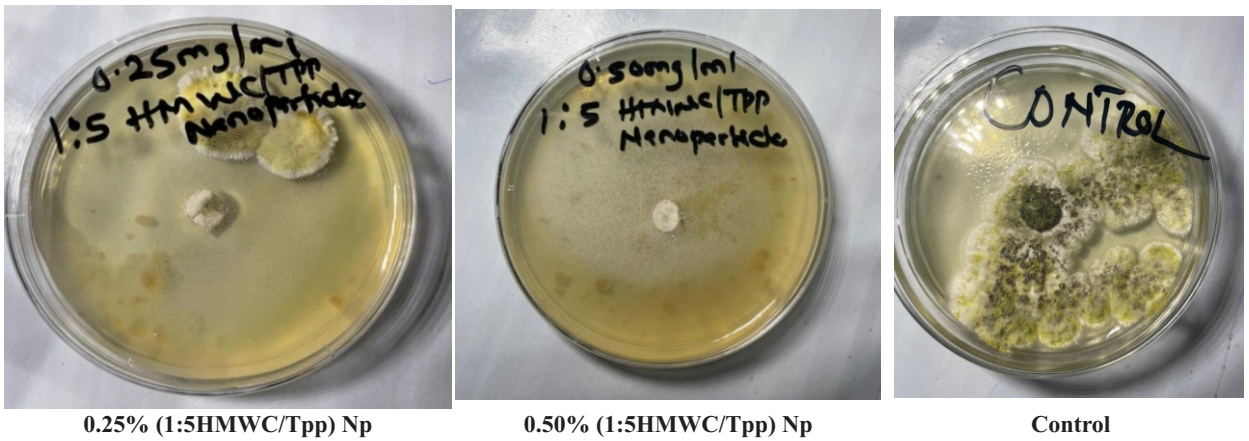
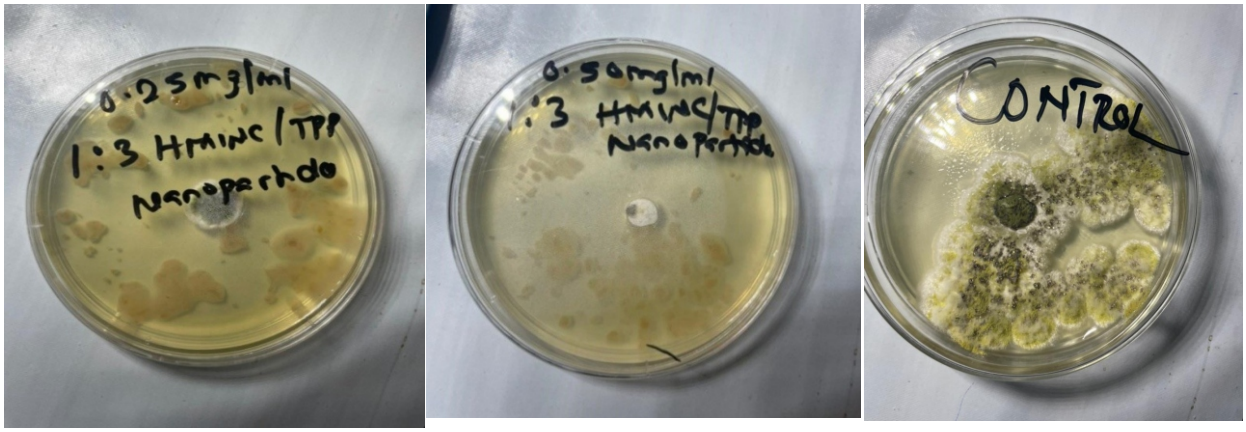
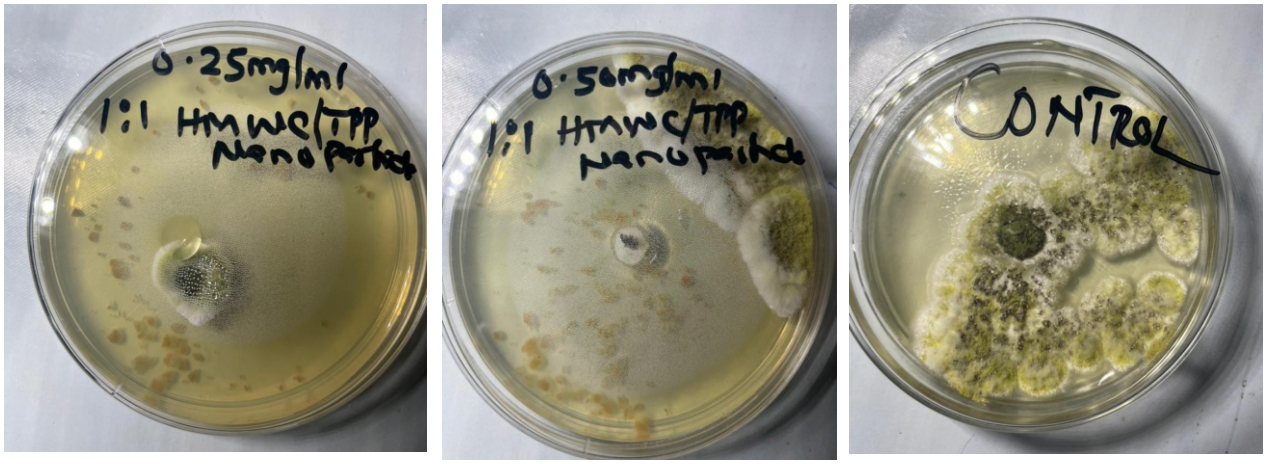


Plate 3: Growth inhibition of *Aspergillus flavus* in different concentration of 711 nm nano-encapsulated chitosan.



0.25% (1:3HMMWC/Tpp) Np **0.50% (1:3HMMWC/Tpp) Np** **Control**

Plate 4: Growth inhibition of *Aspergillus flavus* in different concentration of 468.3 nm nano-encapsulated chitosan



0.25% (1:1HMWC/Tpp) Np **0.50% (1:1HMWC/Tpp) Np** **Control**

Plate 5: Growth inhibition of *Aspergillus flavus* in different concentration of 525.4 nm nano-encapsulated chitosan

DISCUSSION

The results of the synthesized nano-encapsulated chitosan revealed that the particle size was affected by the ratio of chitosan (Cs) concentration to sodium tripolyphosphate (TPP). Also, it was observed that the higher the particle size, the higher the poly disparity index. This trend suggests that the nanoparticle clustered along with the consistency and efficiency of the particle surface. The higher particle size was due to the higher concentration of TPP, as negatively charged phosphate ions react with chitosan amino groups. Hence, the positive surface charge of the nanoparticles decreases. The result is in agreement with the work of Vaezifer *et al.* (2013) and Anan-Raj *et al.* (2015), who both reported that the size of synthesized nano-encapsulated chitosan is affected by the molecular size of the chitosan as well as the ratio of Sodium Tri-poly phosphate to chitosan used. The results are in similar agreement with the reports of Bangun *et al.* (2018), whose results revealed variations in CS and TPP concentrations affect the particle size of nanoparticle suspension.

The antifungal *in-vitro* inhibition test of chitosan revealed that an increase in the concentration of chitosan resulted in a decrease in the growth of *A. flavus*. The results are similar to the report of Contreras-Cortes *et al.* (2019), that the higher the concentration of chitosan, the higher the percentage of inhibition. Nano-encapsulated chitosan percentage fungal inhibition (Table 4) also shows that the range of inhibition was affected by the concentration of the nano-encapsulated chitosan, according to the

incubation period. The result revealed that the lowest concentration of chitosan nanoparticle (0.25%) inhibits *A. flavus* better than chitosan in its free form. The result is similar to the work of Singh *et al.* (2021), who reported that chitosan in its free form has less inhibitory effect than nano-encapsulated chitosan. The result agrees with the earlier findings of Abdel-Aliem *et al.* (2019), who reported that chitosan nanoparticles (COS-NPs) showed excellent antifungal activity against some selected fungi in a dose-dependent manner.

CONCLUSION

Synthesized nano-encapsulated chitosan using the ionic gelation method produced products with 25.4 nm, 468.3 nm, and 711.7 nm particle size in Chitosan and Sodium Tri-polyphosphate ratio-dependent (1:1, 1:3, and 1:5, respectively). The results of chitosan inhibition potential showed that free chitosan concentration of 1.5% and 2.0% have fungicidal properties (100% inhibition), and the lower concentration of 0.5% and 1.0% has fungistatic properties (45% and 75% inhibition, respectively) against *A. flavus*. The inhibition potential of nano-encapsulated chitosan across all the particle sizes at a concentration of 0.50% has fungistatic properties (87.18% inhibition) being the highest in 711.7 nm particle sizes, and if increased, fungicidal properties can be achieved. This study provides evidence that a lower concentration of nano-encapsulated chitosan inhibits *A. flavus* compared to a higher concentration of free chitosan.

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