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Evaluation of antimicrobial activity of chitosan derived from *Aspergillus flavus* strain AF2118 against food and plant pathogens

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Abstract
In recent years, chitosan and its derivatives have been seeking more attention in sustainable agriculture and food safety due to its unique physiochemical and biological characteristics. Therefore, the study aims to evaluate the antimicrobial efficacy of fungal chitosan against plant pathogenic and food spoilage micro-organisms including Gram-positive, Gram-negative bacteria and fungi. The antimicrobial activity of chitosan extracted from *Aspergillus flavus* strain AF2118 in inhibiting the growth of *Staphylococcus aureus* VTCCBAA20, *Escherichia coli* VTCCBAA129, *Pseudomonas aeruginosa* VTCCBAA2 and *Bacillus cereus* VTCCBAA443 were evaluated. Fungal chitosan was effective against *E. coli* and *P. aeruginosa* @ 1000 ppm which exhibits prominent zone of inhibition of 8.00 and 6.33 mm, respectively. The fungal chitosan was also effective in inhibiting the growth of Gram positive bacteria namely, *S. aureus* and *B. cereus* @ 1000 ppm with a maximum clearance zone of 5.33 mm and 2.66 mm, respectively. Chitosan at a concentration of 1000ppm inhibited the growth of *Xanthomonas campestris* with a clearance zone of 1.33 mm. The highest percentage of inhibition of chitosan against fungal pathogens were observed at 3000 ppm for *Rhizoctonia solani* (90.73%) and *Fusarium oxysporum* (76.27%), respectively.

Keywords: Fungal chitosan, *Aspergillus flavus,* Antimicrobial activities, Gram positive bacteria, Gram negative bacteria, plant pathogens

Introduction
Chitosan is a flexible natural hydrophilic polysaccharide made up of 1,4-glycosidic linkages that connect glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) units. It combines a diverse range of physiochemical and biological properties that have attracted interest as possible food preservatives and natural insecticides. Chitosan has wide antibacterial action against gram-positive bacteria, gram-negative bacteria, yeast, and fungi [1].
Antimicrobial chemical compounds employed as food preservatives have a narrow spectrum of effectiveness against some pathogens, which is a severe disadvantage. Furthermore, following ingestion, these chemical antibacterial substances cause some negative effects in the consumer. As a result, a cost-effective bio-preservative with higher antibacterial efficiency and no adverse effects on the consumer is required. Chitosan can be used as a gelling agent. Chitosan can be used as a bio-preservative to extend the storage life of fresh fruits and other packaged food products. Tayel *et al.* [2] determined the anticandidal activity of fungal chitosan extracted from *Mucor rouxii* against three strains of *Candida albicans.* Alshubaily and Al-Zahrani [3] extracted bioactive chitosan polymer from *Cunninghamella elegans* and the antimicrobial effect of chitosan cross-linked ceftriaxone was studied using disc diffusion against three strains of *Staphylococcus aureus*.
Recently, much research has also focused on the antimicrobial properties of chitosan with the possibility of plant protection [4]. Several studies have been conducted on the antimicrobial activities of chitosan against plant pathogens such as *Xanthomonas* sp., *Erwinia carotovora*, *Fusarium graminearum*, *Verticillium dahliae*, *Alternaria solani*, *Botryti cinerea*, *C. orbiculare*, *Sclerotinia sclerotiorum*, *F. oxysporum*, and *Phytophthora capsici* [5-6]. Chitosan is advantageous over other types of antimicrobial agents due to its higher antimicrobial activity, a broader spectrum of activity, and lower toxicity toward mammalian cells. The antimicrobial activity of chitosan mainly depends on its degree of deacetylation and molecular weight. Chitosan controls pathogenic microorganisms by restricting growth, germination,

sporulation, and reducing spore viability. The primary mechanism involved in the antibacterial activity is the electrostatic interaction of chitosan with the cell wall, plasma membrane, and cytoplasmic constituents. It is generally assumed that the cationic nature of chitosan is due to the presence of the positively charged NH^{3+} groups of glucosamine, which serves as the fundamental factor contributing to its interaction with the negatively charged microbial cell surface that finally results in impairment of vital bacterial activities [7].

The purpose of the present study was to examine the in vitro antimicrobial effects of fungal chitosan extracted from *Aspergillus flavus* strain AF2118 against food spoilage and plant pathogenic microorganisms.

Materials and Methods

Microbial Sample Preparation

The test organisms, *Staphylococcus aureus* VTCCBAA20, *Escherichia coli* VTCCBAA129, *Pseudomonas aeruginosa* VTCCBAA 2 and *Bacillus cereus* VTCCBAA443, were obtained from the National Centre for Veterinary Type Cultures of ICAR-National Centre on Equines, Hisar, Haryana. The plant pathogenic cultures of *Fusarium oxysporum*, *Xanthomonas campestris,* and *Rhizoctonia solani* were obtained from the Department of Plant Pathology, CCS HAU, Hisar. The fungal cultures were maintained on potato dextrose agar (PDA) slants by regular sub-culturing and stored at 4 °C until further use.

Extraction of chitosan from mycelia of *Aspergillus flavus* strain AF2118

The extraction of chitosan from *Aspergillus flavus* strain AF2118 isolated from canal water was determined using a modified method [8]. After cultivation, fungal mycelia were harvested by filtration through Whatman filter paper No. 1, followed by washing with distilled water and drying at 65 °C to a constant weight. Dry fungal mycelia were finely ground and suspended with 1.0 M NaOH solution (1: 30 w/v) in 100ml Erlenmeyer flasks, followed by autoclaving at 121 °C, 15 lbs of pressure for 15 minutes. The alkali-insoluble materials were collected by filtering the autoclaved slurry through Whatman filter paper No.1 followed by washing with distilled water. The residues were further extracted using 1.0 M HCl (1: 40 w/v) by autoclaving at 121 °C, 15 lbs of pressure, for 15 minutes. The extracted slurry containing the chitosan was centrifuged at 10,000 rpm for 10 minutes and the acid insoluble fraction was discarded. The pH of the supernatant fluids was adjusted between 10 and 12 using 2.0 M NaOH to precipitate chitosan, followed by centrifuging the solution at 10,000 rpm for 15 minutes. The precipitated chitosan was washed with distilled water, 95% ethanol (1: 20 w/v) and acetone (1: 20 w/v), respectively, followed by drying at 60 °C to a constant weight.

Preparation of chitosan stock solution

To begin, 1.0% (v/v) acetic acid was prepared for dissolving fungal chitosan. The purified chitosan powder (0.1 g) was weighed accurately and dissolved in 100 ml of 1.0% (v/v) acetic acid to get a concentration of 1000 ppm. The flask was shaken several times to get the solution well mixed. The pH of the medium was adjusted to 5.6 and the solution was autoclaved at 121 °C, 15 psi for 20 minutes. Then various concentrations of chitosan solution, such as 50, 100, 500, and 1000 ppm, were prepared from the above stock solution.

Evaluation of chitosan for antimicrobial activity

The antimicrobial activity of fungal chitosan was determined against different pathogenic microorganisms like *Staphylococcus aureus* VTCCBAA20, *Escherichia coli* VTCCBAA129, *Pseudomonas aeruginosa* VTCCBAA2, *Bacillus cereus* VTCCBAA443 and *Xanthomonas campestris* by the filter paper disc diffusion method [9]. The effects of chitosan on the growth of fungi were determined using the poisoned substrate technique [10].

Evaluation of chitosan for antimicrobial activity against food pathogens

A loopful of each bacterium grown isolated in LB agar plates for 18 to 24 hours was inoculated separately into 50 ml of sterilised LB broth in 100 ml flasks and kept in an orbital incubator shaker at 37 °C at 140 rpm. 1.0 ml of overnight grown bacterial cultures (*Staphylococcus aureus* VTCCBAA20, *Escherichia coli* VTCCBAA129, *Pseudomonas aeruginosa* VTCCBAA2, *Bacillus cereus* VTCCBAA443) were spread separately on LB agar plates using sterile glass spreaders and allowed to dry for 10 minutes. Then a sterilised filter paper disc was dipped in different concentrations of chitosan and placed on the surface of agar media which had previously been incubated with the test organism with the aid of sterile forceps. The plates were incubated at 37°C for 48 hours in an incubator. Acetic acid, 1.0%, was used as a control in all the species. All the experiments were performed in triplicate in order to confirm reproducibility and reliability. The zone of inhibition was measured in millimetres.

Evaluation of chitosan for antimicrobial activity against plant pathogens

The plant pathogenic cultures such as *Xanthomonas campestris, Fusarium oxysporum,* and *Rhizoctonia solani* were tested for the antimicrobial activity of chitosan. A loopful of *Xanthomonas campestris* from 18 to 24 hours of grown isolated colonies was inoculated into 50 ml of sterilised nutrient broth in 100 ml flasks and incubated at 28 2 °C in an orbital shaker at 140 rpm. From the overnight grown cultures, 1000l were taken using a micropipette and spread on nutrient agar plates using a sterile glass spreader, followed by drying for 10 minutes. A sterilised filter paper disc was dipped in different concentrations of chitosan and was placed on the surface of the media aseptically using sterile forceps. The plates were incubated at 28 °C for 48 h in an incubator. 1.0% acetic acid was used as a control. The zone of inhibition was measured in millimetres.

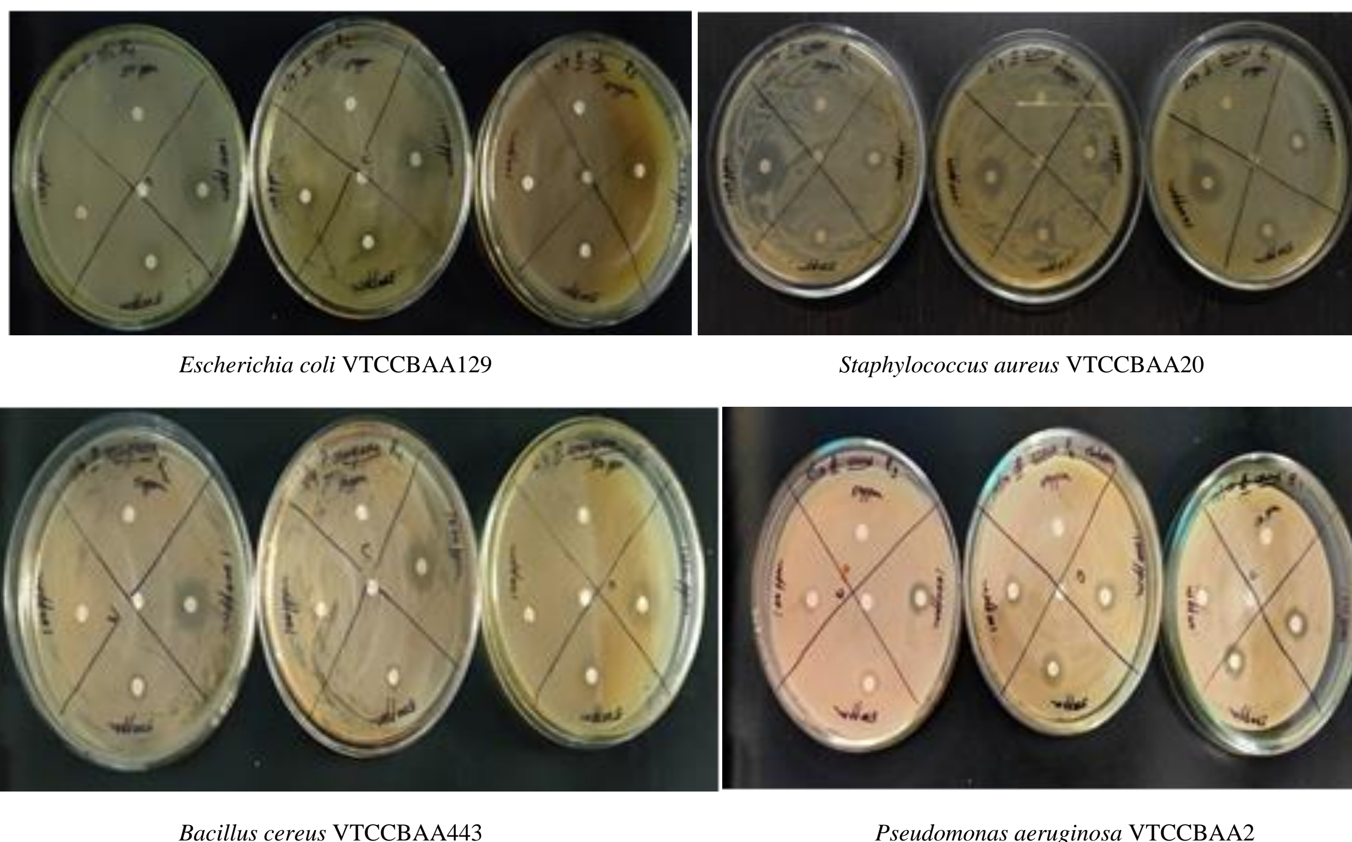
The antifungal properties of chitosan against fungal pathogens were assessed separately for each fungus using PDA plates amended with different concentrations of chitosan such as 1000, 2000, and 3000 ppm. Potato Dextrose agar media (60 ml) was taken in a 250 ml Erlenmeyer flask and sterilised in an autoclave at 121°C for 15 psi for 15 minutes. Chitosan solutions of different concentrations were added to each flask containing PDA media and mixed gently. The media was then poured into petri plates (20 mL each), followed by solidification. A fungal mycelial disc was placed on the petri plates containing chitosan in different concentrations (1000, 2000, and 3000 ppm). A control was maintained with 1.0% acetic acid without amending chitosan. Four replications were

maintained against each concentration and control for each fungus. The petri plates were then incubated at 28±2 °C in a BOD incubator.
The radial growth was measured when the fungi in the control treatment reached the edge of the plate. The percentage of inhibition of radial growth relative to control was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Statistical analysis
The results were statistically analysed using analysis of variance techniques (ANOVA) as applied to the completely randomised design (CRD) described by Panse and Sukhatme [11].

Results and Discussion
Evaluation of fungal chitosan for antimicrobial activity against food pathogens
The effectiveness of chitosan extracted from *Aspergillus flavus* strain AF2118 in inhibiting the growth of *Staphylococcus aureus* VTCCBAA20, *Escherichia coli* VTCCBAA129,*Pseudomonas aeruginosa* VTCCBAA2 and *Bacillus cereus* VTCCBAA443 was determined by measuring the clearance zone around the disc.In the present study, the zone of inhibition test revealed mainly two types of results: a clearance zone around the disc represented a bacteriostatic action of fungal chitosan, and a disc without an inhibition zone indicated the absence of inhibitory activity (Fig.1; Plate 1). The observations recorded in this study estimated that fungal chitosan had antibacterial activity against both Gram positive (*Staphylococcus aureus* VTCCBAA20 and *Bacillus cereus* VTCCBAA443) and gram negative bacteria (*Escherichia coli* VTCCBAA129 and *Pseudomonas aeruginosa* VTCCBAA2). The fungal chitosan was more effective at inhibiting gram negative bacteria as compared to gram positive at higher concentrations (1000 ppm). All the test organisms were grown in control plates with 1.0% acetic acid without chitosan as well as at a 50 ppm concentration of fungal chitosan.



Escherichia coli VTCCBAA129 *Staphylococcus aureus* VTCCBAA20

Bacillus cereus VTCCBAA443 *Pseudomonas aeruginosa* VTCCBAA2

Plate 1: Effect of different concentrations of fungal chitosan against food pathogens at 50, 100, 500 and 1000ppm along with control (c)

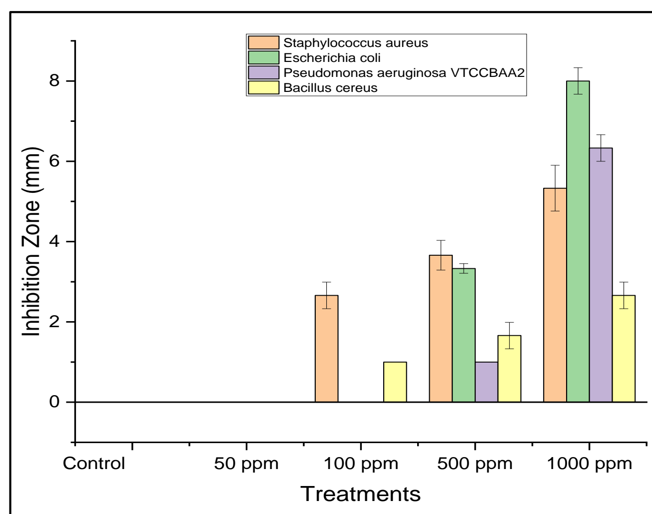


Fig 1: Evaluation of fungal chitosan for antimicrobial activity against food pathogens

The *in vitro* results showed that the antibacterial effects of fungal chitosan against *Escherichia coli* VTCCBAA129 were 3.33 and 8.00 mm at 500 ppm and 1000 ppm, respectively. In the case of *Pseudomonas aeruginosa* VTCCBAA2, the zones of inhibition at concentrations of 500 and 1000 ppm were found to be 1.0 and 6.33 mm, respectively. At 100 ppm of chitosan solution, both the Gram-negative bacteria were fully grown. The antibacterial activity of chitosan against *Staphylococcus aureus* VTCCBAA20 and *Bacillus cereus* VTCCBAA443 at 1000 ppm was estimated to be 5.33 and 2.66 mm, respectively (Table 1).

Table 1: Evaluation of *Aspergillus flavus* strain AF2118 chitosan for antimicrobial activity against food pathogens

Treatments	Inhibition Zone (mm)			
	Staphylococcus aureus VTCCBAA20 G+	*Escherichia coli* VTCCBAA129 G-	*Pseudomonas aeruginosa* VTCCBAA2 G-	*Bacillus cereus* VTCCBAA443 G+
Control (1% Acetic acid)	0 (0.701)*	0 (0.701)	0 (0.701)	0 (0.701)
50 ppm	0 (0.701)	0 (0.701)	0 (0.701)	0 (0.701)
100 ppm	2.66±0.33 (1.772)	0 (0.701)	0 (0.701)	1±0.00 (1.227)
500 ppm	3.66±0.37 (2.114)	3.33±0.12 (1.933)	1±0.00 (1.227)	1.66±0.33 (1.463)
1000 ppm	5.33±0.57 (2.791)	8±0.45 (2.91)	6.33±0.33 (1.953)	2.66±0.33 (1.772)
CD at 5%	0.325	0.345	0.116	0.217

*Values in parenthesis are mean of square root transformation

The fungal chitosan exhibited a prominent antibacterial effect against gram-positive bacteria, namely, *Staphylococcus aureus* VTCCBAA20 and *Bacillus cereus* VTCCBAA443. The clearance zone was measured at a 100 ppm concentration of fungal chitosan. The antibacterial activity was most exhibited against *Staphylococcus aureus* VTCCBAA20 with a clearance zone of 2.66, 3.66, and 5.33 mm at concentrations of 100, 500, and 1000 ppm, respectively. The zone of inhibition exhibited for *Bacillus cereus* VTCCBAA443 was 1.0, 1.66, and 2.66 mm at concentrations of 100, 500, and 1000 ppm, respectively.

The antimicrobial activity of chitosan has been investigated against many bacteria, filamentous fungi and yeasts [12-14]. Chitosan had bacteriostatic effect against bacteria related to many foodborne illness including *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholerae*, *Bacillus subtilis*, *B. cereus*, *Bacteroides fragilis* and *Shigella dysenteriae* [15].

This study agrees with the findings of Jebur *et al.* [16], who examined the antibacterial activity of chitosan derived *Aspergillus oryzae* HQ285580 with an inhibition zone against *Staphylococcus aureus* (8.0 mm), *Escherichia coli* (2.0 mm) and *Bacillus subtilus* (2.0 mm). The studies conducted by Chung and Chen [17] and Chang *et al.* [18] suggested that the inactivation of *E. coli* and *S. aureus* by chitosan was due to the electrostatic interaction between the polycationic structure and anionic components of their cell surface, separation of the cell wall from its plasma membrane, and destruction of the cell membrane. Chitosan derived from the marine Penicillum spinulosum MH2 I demonstrated antibacterial activity against S. aureus with a zone of inhibition of 3.5 mm [19]. Omogbai and Ikenebomeh [20] reported that the chitosan derived from *Rhizopus oryzae* inhibited the growth of *Escherichia coli* (83.2%), *Salmonella typhi* (67.9%), *Pseudomonas aeruginosa* (63.8%), and *Bacillus subtilis* (62.4%) at a concentration of 50mg/L in 24 hours. In their studies, Aleanizy *et al.* [21] demonstrated in their studies that the treatment with chitosan nanoparticles at 1000 ppm concentration resulted in disruption of the permeability of the outer membrane of *P. aeruginosa.*

In this study, we further found that gram-negative bacteria

were more susceptible to fungal chitosan than gram-positive bacteria at higher concentrations. This difference in sensitivity is largely contributed to the structures of their cell envelopes. The gram-negative cell wall is thinner and consequently more susceptible to fungal chitosan than the gram-positive bacteria (90% interpeptide cross linkages in the cell wall).

Evaluation of fungal chitosan for antimicrobial activity against plant pathogen

The antibacterial activity of chitosan against *Xanthomonas was recorded as* an inhibition zone of 1.33 mm at 1000 ppm of chitosan. There was no clearance zone observed at 50, 100, and 500 ppm of fungal chitosan.

In agriculture, pathogens cause various kinds of plant diseases that result in reduced plant growth, inferior product quality, lower yields, and huge economic losses. Hence, plant diseases should be controlled to maintain the quality of agricultural products. The antimicrobial properties of chitosan were also investigated against the plant pathogenic strains of *Xanthomonas campestris*, *Fusarium oxysporum* and *Rhizoctonia solani*.

The antibacterial activity of chitosan against *Xanthomonas was recorded as* an inhibition zone of 1.33 mm at 1000 ppm of chitosan. The studies conducted by Wang *et al*. [7] stated that the antibacterial activity of chitosan against *Xanthomonas* strains exhibited a reduction percentage of 61.88% at 0.10 mg/mL of chitosan. They also indicated that chitosan at higher concentrations increased the release of DNA and RNA from the cell. Transmission electron microscopic studies revealed that chitosan caused alterations in protoplast concentration and cell surface morphology of *Xanthomonas*.

The antifungal properties of chitosan against pathogenic fungi were assessed for each fungus at various concentrations of fungal chitosan, such as 1000, 2000, and 3000 ppm. The fungal chitosan significantly decreased the radial growth of both fungi (Table 2). The mycelial growth of fungi was reduced on media amended with fungal chitosan at 2000 ppm and 3000 ppm (Plate 2 A and B). However, the maximum growth was observed in control plates with 1.0% acetic acid as well as at a 1000 ppm concentration of chitosan. The average reduction in radial growth of fungi was found to be higher at 3000 ppm (76.27%) and lowest (54.32%) at 2000 ppm for *Fusarium oxysporum* when compared with control (1% acetic acid). Similar observations were measured for *Rhizoctonia solani,* with a higher percentage of inhibition at 2000 ppm (73.33%) and 3000 ppm (90.73%). Among the fungi tested, *Rhizoctonia solani* was observed to be more sensitive to fungal chitosan at higher concentrations as compared with *Fusarium oxysporum.* The highest percentage of inhibition was recorded at 3000 ppm for both fungi (*Rhizoctonia solani* (90.73%) and *Fusarium oxysporum* (76.27%), respectively).

In this study, the average reduction in radial growth of fungi was found to be higher at 3000 ppm (76.27%) and least (54.32%) at 2000 ppm for *Fusarium oxysporum* (Table 2). According to the studies conducted by Zhong *et al*. [22], chitosan had a significant antifungal effect on *Fusarium oxysoporum* with 85% inhibition at higher concentrations. Palma Guerrero *et al*. [23] discovered that chitosan penetrates the conidia of F. oxysporum by an energy-dependent process and causes ultrastructural alterations such as cytoplasm disorganization, retraction of the plasma membrane, and loss of intracellular content (15 minutes).

The data in table 2 reveals that *Rhizoctonia solani* was assessed with a higher percentage of inhibition at 2000 ppm (73.33%) and 3000 ppm (90.73%). The studies conducted by Mohammed *et al*. [24] stated that chitosan at 1% concentration completely inhibited the growth of *R. solani* by the poisoned food technique in PDA medium. Similar observations were also reported in the studies conducted by Liu *et al.* [25] (2012) and Bautista-Banos *et al.* [26] (2004), with 60–91% inhibition in radial growth of *R. solani* and *F. oxysporum* at higher concentrations of chitosan.

Table 2: Effect of chitosan on radial growth of fungal pathogens

Treatments	Inhibition (%)	
	Fusarium oxysporum	***Rhizoctonia solani***
Control (1% Acetic acid)	0.00 (0.984)	0.00 (0.959)
1000µg/ml	0.00 (0.984)	0.00 (0.959)
2000 µg/ml	54.32±0.24 (47.48)	73.33±0.64 (38.91)
3000 µg/ml	76.27±0.53 (60.85)	90.73±0.76 (72.46)
CD at 5%	2.41	3.24

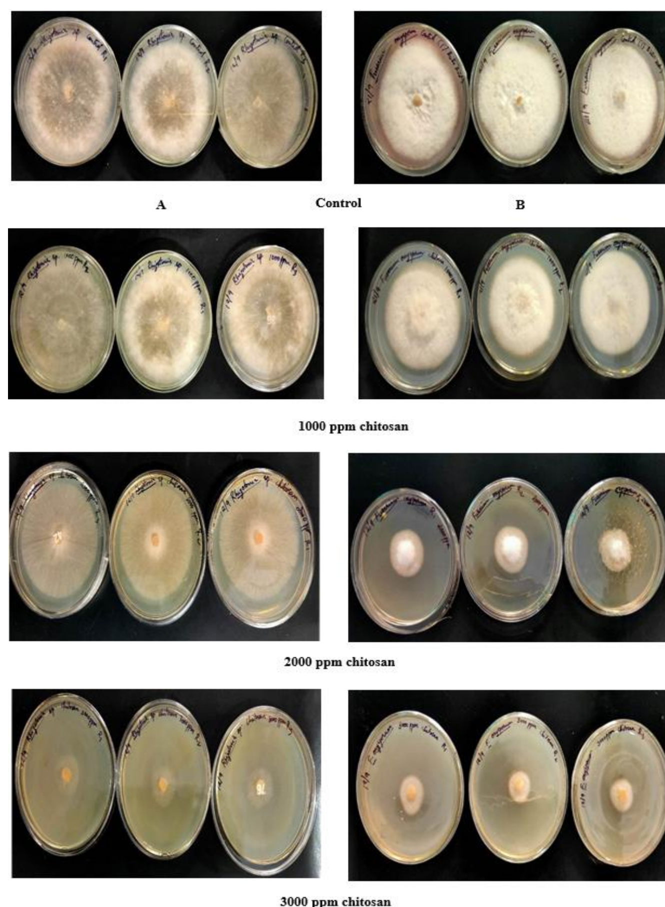


Plate 2: Effect of different concentrations of fungal chitosan on radial growth of A. *Rhizoctonia solani* and B. *Fusarium oxysporum*

Conclusion

In the present study, chitosan extracted from *Aspergillus flavus* strain AF2118 showed antimicrobial activity against food spoilage bacteria and plant pathogenic microorganisms. The fungal chitosan was more effective at inhibiting gram negative bacteria (*Escherichia coli* VTCCBAA129 and *Pseudomonas aeruginosa* VTCCBAA2) as compared to gram

positive (*Staphylococcus aureus* VTCCBAA20 and *Bacillus cereus* VTCCBAA443) at higher concentrations (1000 ppm). Nowadays, farmers are reluctant to use hazardous chemical pesticides for plant protection since there is an increasing awareness of sustainable agricultural systems. From the above results, fungal chitosan exhibited antibacterial activity against the growth of *Xanthomonas campestris* with a clearance zone of 1.33 mm at 1000 ppm and antifungal activity against *Rhizoctonia solani* (90.73%) and *Fusarium oxysporum* (76.27%). Moreover, the fungal chitosan is non-toxic, organic in nature, and economical and could be exploited as an eco-compatible material for management of pathogenic diseases. Hence, the present study reveals the potential application of biological chitosan as a food preservative and bio pesticide in the future.

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