GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Evaluation of antifungal efficacy of chitosan against *Aspergillus fumigatus* of stored rice (*Oryza sativa*)

Mariam Bukola Aremu [1, *], Matthew Omoniyi Adebola [2], Evans C Egwim [3] and Muhammadu Tajudeen Salaudeen [4]

[1] *Durable Crop Research Department, Nigeria Stored Products Research Institute, Km 3, Asa Dam Road, Ilorin, Kwara State, Nigeria.*
[2] *Department of Plant Biology, School of Life Sciences, Federal University of Technology, Minna, Niger State, Nigeria.*
[3] *Department of Biochemistry, School of Life Sciences, Federal University of Technology, Minna, Niger State, Nigeria.*
[4] *Department of Crop Production and Protection, School of Agriculture and Agricultural Technology, Federal University of Technology, Minna, Niger State, Nigeria.*

GSC Biological and Pharmaceutical Sciences, 2023, 22(03), 204–209

Publication history: Received on 05 February 2023; revised on 21 March 2023; accepted on 24 March 2023

Article DOI: https://doi.org/10.30574/gscbps.2023.22.3.0113

Abstract

Contamination of stored rice with fungal pathogens results in rice of poor quality and economic value, and may also have harmful effects on human and animal health. This study aimed at evaluating the antifungal efficacy of chitosan against *Aspergillus fumigatus* of stored rice (*Oryza sativa*). Four forms of chitosan were used in this study viz; Purchased Low, Medium and high molecular weight chitosan and chitosan synthesized from Crab Shell using deproteinization, demineralization, decolouration and deacetylation. Degree of deacetylation of the synthesized chitosan was determined using Fourier transform infrared (FTIR) analysis. The *in vitro* antifungal activity of all forms of chitosan were determined using food poisoning method against *Aspergillus fumigatus* isolated from stored rice seeds using agar plate methods. The percentage inhibition of mycelial radial growth *Aspergillus fumigatus* by the different forms of chitosan was determined. Fourier Transfrom InfraRed Spectra of synthesized chitosan showed major absorption bands from 3444.72, 2966.17, 2512.60, 2144.84, 1429.74, 1258.12, 1160.05, 1025.2, 869.92, 710.50, 608.40 to 559.36. Absorption peak at 559 was assigned to glucopyranose ring in the chitosan matrix. Degree of deacetylation of synthesized chitosan was at 98.6% degree. Highest mycelia radial growth of 8.00 cm was recorded in control, while 2.00, 0.00, 5.23, and 1.33cm were recorded in 2.0% concentration of High Molecular Weight Chitosan (HMWC), Medium Molecular Weight Chitosan (MMWC), Low Molecular Weight Chitosan (LMWC) and Chitosan synthesized from Crab shell (CSCS) treatment respectively. The result of the percentage inhibition reveals that *A. fumigatus* had 100% inhibition in 1.5% HMWC and 2.0% MMWC while 85% inhibition in 2.0% CSCS which were significantly different from 2.0% LMWC with 36.5% inhibitions. Results obtained from this study has shown the effectiveness of chitosan as an effective antifungal agent which can improve and maintain the quality of stored rice and ensure a safer environment due to its nontoxic and environmental friendly properties.

Keywords: Crab Shell; Fungal; Inhibition; Pathogen; Synthesized

1. Introduction

Rice (*Oryzae sativa* L.) is an important cereal, grown around the world, especially in Africa and Asia [1]. Rice represents the main food source for about half of the world's population [1]. In Nigeria, it is one of the top sustaining food after sugarcane and maize [1]. According to [2], while the amount of harvested rice is increasing annually, consumer intake has gradually declined, decreasing by half between 1985 and 2015. Consequently, this has led to harvested rice being

* Corresponding author: Mariam Bukola Aremu; Email:mariambukola036@yahoo.com

stored for longer periods to ensure year-round availability. During storage, rice is susceptible to contamination by various fungal species that can reduce its quality and market value. *Aspergillus, Fusarium* and *Penicillium* species have been reported to be among the prominent fungal genera of stored rice seed [3]. The occurrence of these pathogens in stored rice has put a lot of pressure on farmers and agriculturalists with the major challenge coming from ways to protect this staple crop from this menace. The use of synthetic fungicides has been the primary method of controlling these fungal pathogens [4]. Although this has been effective, the disadvantages to widespread use of these synthetic products are significant. They include attendant pollutants effects on the environment, cost of these chemicals, development of resistance in fungal pathogens and concerns about chemical residues on food products meant for consumption. This has shifted focus to newer and safer approaches in which crop products are free of chemical residues, the environment is safe and treatment means cost effective.

Introduction of new biofungicides (Chitosan) is an alternative solution to solve this problem as it has been used in crop production and protection of various fruits and vegetables [5]. Chitosan is the second most renewable carbon source after lignocelluloses biomass [6]. In rice protection, treatments with chitosan have been shown to prevent the growth of several pathogenic microorganisms including the rice sheath blight pathogen, *Rhizoctonia solani*, and *Xanthomonas* in the ornamental plant *Euphorbia pulcherrima*, *Xanthomonas axonopodis* pv. *Poinsettiicola* [7]. Therefore evaluating the antifungal efficacy of chitosan against *Aspergillus fumigatus* of stored Rice (*Oryza sativa*) is important in achieving integrated pest management.

2. Material and methods

2.1. Collection of sample

Three different molecular weight chitosan (Low molecular weight chitosan (LMWC) (MW 50 kDa; 75–85% deacetylated), Medium molecular weight chitosan (MMWC) (MW 400 kDa; 75–85% deacetylated) and High molecular weight chitosan (HMWC) (MW 760 kDa; ≥ 85%)) were purchased from Chitin-Chitosan BioChemika and Sigma- Aldrich Company USA. Crab shells were also obtained from sea shores in Warri, Delta State Nigeria. The growth medium Potato Dextrose Agar (PDA) and Potato Dextrose Broth were also sourced from Bristol Scientific, Lagos, Nigeria.

2.2. Chitosan Synthesize from Crab Shell

Crab (*Callinectes amnicola*) shell waste were washed and dried in hot air oven at 60 ^{o}C for 24 hrs. Dried shell waste were packed in polyethylene bag and stored at 4 ^{o}C. Dried shells were pulverized manually using mortar and pestle. The modified extraction procedure of [8] was followed which included basic steps of deproteinization, demineralization, decolouration and deacetylation.

Pulverized shells were deproteinized by treating with 3.5% (w/w) NaOH solution for 2 hrs at 65^{o}C with constant stirring at a solid to solvent ratio of 1:10 (w/v), demineralized with 1N HCL for 30 min at ambient temperature in a solid to solvent ratio of 1:15 (w/v) for 15 min and decolourized with acetone for 10 min and dried for 2 hrs under hood, followed by bleaching with 0.32 % (v/v) solution of sodium hypochloride (containing 5.25% available chlorine). After each step, the chitin was filtered, washed with distilled water to neutral pH. Chitin deacetylation was carried out at 15 psi/121^{o}C using 50 % sodium hydroxide (NaOH) solution for 15 min. The samples were filtered off, washed with distilled water to neutral pH and dried in an oven at 60 ^{o}C for 24 hrs.

2.3. Determination of the degree of deacetylation

To determine the degree of deacetylation of the synthesized chitosan, Fourier transform infrared (FTIR) analysis was done. Chitosan solution was prepared in potassium bromide (KBr) as a pellet under 1:99 ratio of chitosan sample to KBr, the sample mixture was then subjected to the infrared (IR) radiation spectroscopy machine (Model-ABB FTLA 2000-100 Quebec, Canada) at a resolution limit of 16 cm^{-1}[9]. The degree of deacetylation of the chitosan was determined from IR results based on the ratio between peak areas at wavelength 1655cm^{-1} and 3450cm^{-}

2.4. Isolation of *Aspergillus fumigatus*

Aspergillus fumigatus was isolated from stored rice seeds using agar plate methods and identified using fungal family of the world mycological monograph [10], the identified cultures was preserved in PDA slants for further use.

2.5. Preparation of chitosan solution

Four types of chitosan (HMWC, MMWC, LMWC and CSCS) were used for the preparation of the solution. Concentrations of 0.5, 1.0, 1.5, and 2.0g of all the four chitosan were weighed and dissolved in 100 ml sterile water containing 0.5 ml

(v/v) glacial acetic acid using an overhead stirrer. The pH of the solution was adjusted to 5.6 by adding either 1N NaOH or 1N HCl depend on the pH reading, using a digital pH meter [11].

2.6. Inhibition of mycelial radial growth of *A. fumigatus*

The *in vitro* antifungal activities of all the chitosan were determined using food poisoning method. A disc (6 mm diameter) each was taken from the pure cultures of *A. fumigatus* using 6mm diameter cork borer and inoculated at the centre of each petri dish containing PDA and chitosan solutions at 0.5, 1.0, 1.5 and 2.0%. Petri dish containing PDA with sterile distilled water with glacial acetic acid was used as control. The plates were in triplicates and incubated at laboratory temperature (28±2°C). Daily mycelial radial growth was measured for 5 days. (Zahid, 2014) The percentage inhibition of mycelial radial growth was calculated using the formula described by Al-Hater [12].

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

Where R1 = mycelial growth in control plates, R2 = mycelial growth in treated plates

3. Results and discussion

The Fourier Transform InfraRed (FTIR) spectrum of the Chitosan showed major absorption bands ranges from 3444.72, 2966.17, 2512.60, 2144.84, 1429.74, 1258.12, 1160.05, 1025.2, 869.92, 710.50, 608.40 to 559.36. (Fig 1). The absorption peak at 559 was recognized due to the anti-symmetric stretching vibration of C-O-C bridges and assigned to glucopyranose ring in chitosan matrix. The result of the banding pattern was supported by the report of [13] whose FTIR spectrum band is between 3430 to 599cm^{-1}.The protocol used for the extraction yield chitosan with 98.6% degree of deacetylation, the result was similar to the work of [8, 14]. They both reported different protocol for the synthesis of chitosan and validate that the best method that produces the highest degree of deacetylated chitosan is deproteinization, demineralization, decolourization and deacetylation (DPMCA)

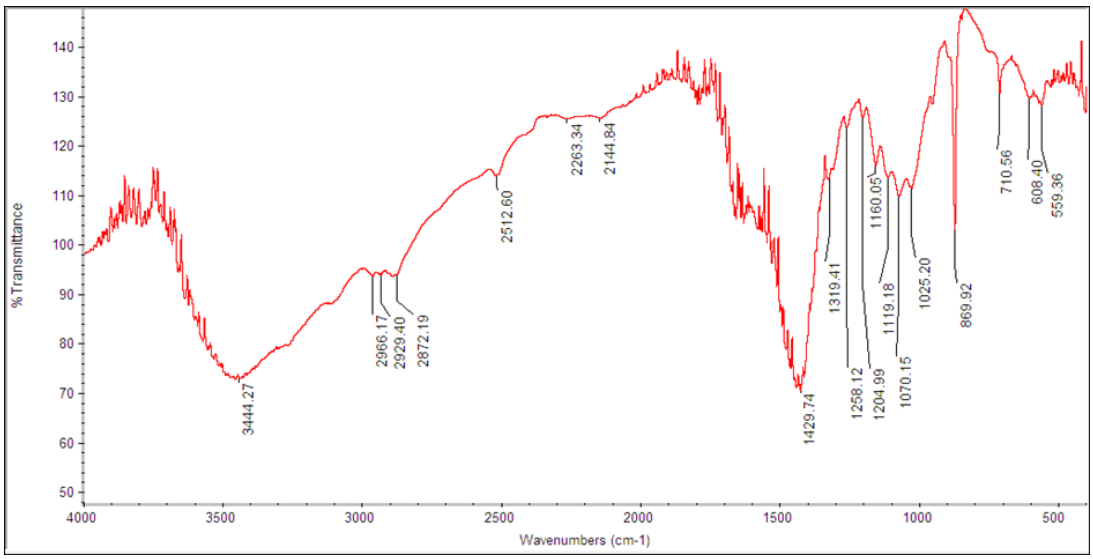
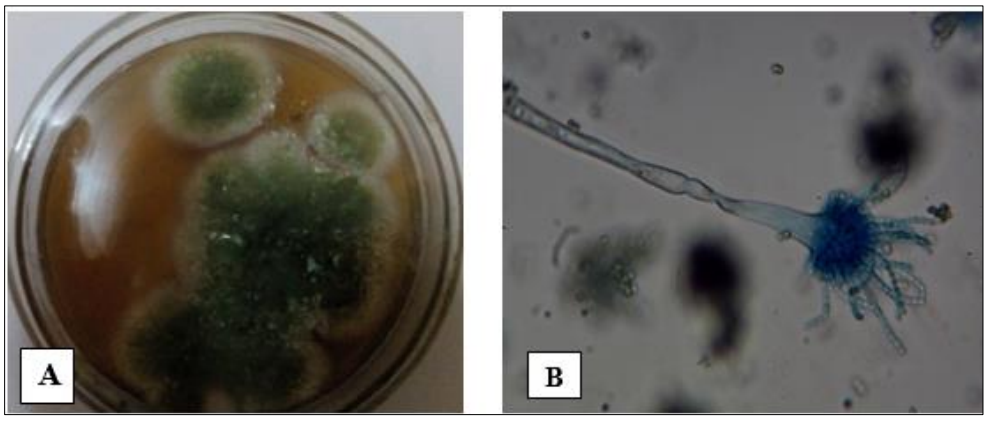


Figure 1 Fourier Transforms Infrared (FTIR) spectra graph of Synthesize Chitosan for determination of DDA

3.1. Isolation and Identification of *Aspergillus fumigatus*

The morphological identification of *A. fumigatus* was dark green colony with whitening powdery edge and the reverse side is cream while the microphotography shows septate hyphae, unbranched conidiophores with secondary branches metulas. Sterigmata bears round conidia in chain (Fig 2)



A: Pure culture of *Aspergillus fumigatus* B: Photomicrograph of *A. fumigatus*

Figure 2 Morphological and Microscopic Identification of *A. fumigatus*

3.2. Mycelia Radial Growth Inhibition of *A. fumigatus*

The highest mycelia radial growth of 8.00cm was recorded in control, while 2.00, 0.00, 5.23, and 1.33cm was recorded in 2.0% concentration of HMWC, MMWC, LMWC and CSCS treatment respectively (Table 1). The results of chitosan inhibition potential showed that chitosan solution at concentration of 1.5% and 2.0% has fungistatic properties against *A. fumigatus*. The result also reveal that the mycelia radial growth inhibition is dose dependent and is in agreement with the report of [15], that the higher the concentration, the lower the mycelia radial growth.

Table 1 Mycelia Radial Growth Inhibition of *A. fumigatus*

Chitosan Concentration	Mean Growth of *A. fumigatus* (cm)			
	HMWC	MMWC	LMWC	CSCS
0/control	8.00b±.06	8.00c±.06	8.00e± .06	8.00e± .06
0.5	0.00a±.00	4.60b±.06	5.80b± .12	6.00b± .06
1.0	1.83a±.17	3.93b±.07	4.70b± .06	5.40b± .06
1.5	0.00a±.00	1.63a±.03	4.83b± .17	3.50b± .20
2.0	2.00a±.00	0.00a±.00	5.23b±.19	1.33a± .17

3.3. Percentage Inhibition of *A. fumigatus*

The result of the percentage inhibition reveals that *A. fumigatus* had 100% inhibition in 1.5% HMWC and 2.0% MMWC while 85% inhibition in 2.0% CSCS which were significantly different from 2.0% LMWC with 36.5% inhibition. (Fig 3). The result is similar to the work of [16] that high molecular weight is the most effective. It was also observe that the percentage inhibition of *A. fumigatus* in 2.0% chitosan synthesized from crab shell (CSCS) was not significantly different from that of HMWC and MMWC, hence, chitosan can be synthesized by individual, stakeholders and companies in the Country instead of importing.

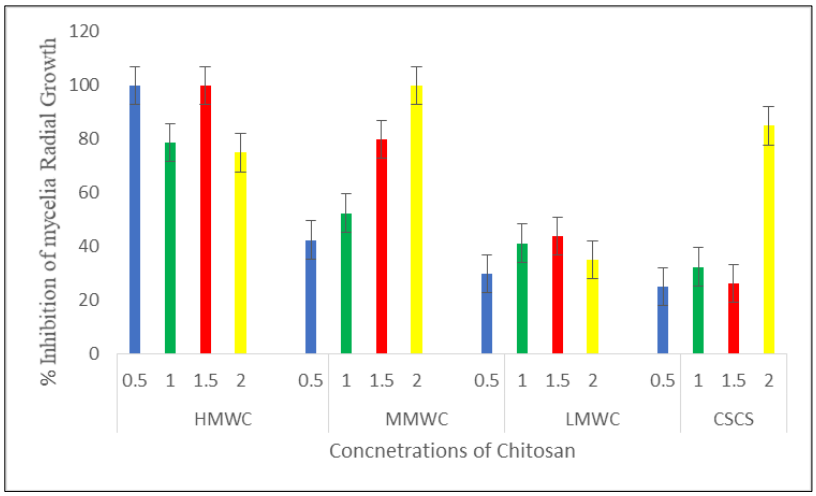
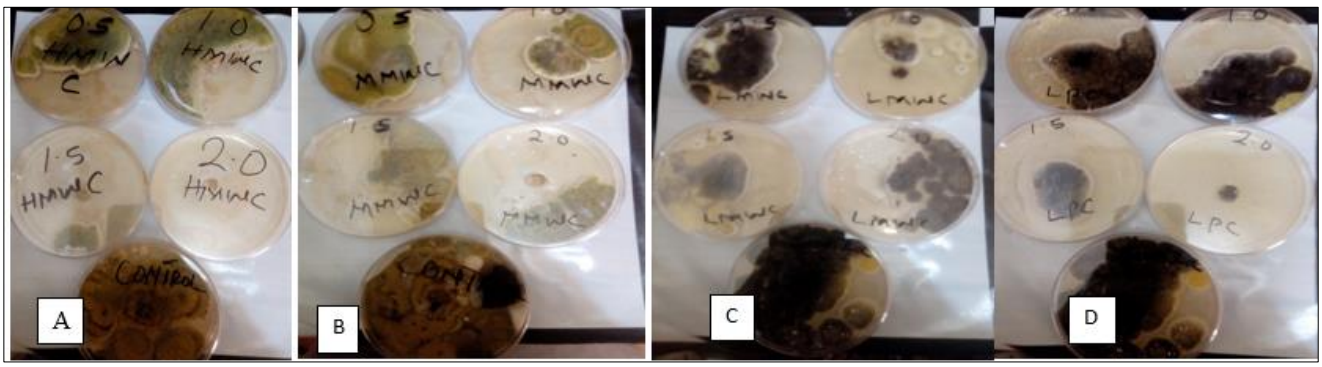


Figure 3 Percentage Inhibition of Mycelia Radial Growth of *A. fumigatus* in different concentrations of Chitosan

The pictorial representation of growth mass of *A. fumigatus* in different concentration of the chitosan (HMWC, MMWC, LMWC and CSCS) (Fig 4) showed that the control plate has the highest growth mass. Growth decrease with increase in concentration of the chitosan in treated plates.



A: High (MW) Chitosan	B: Medium (MW) Chitosan	C: Low (MW) Chitosan	D: Chitosan synthesized from Crab Shell

Figure 4 Mycelial Radial Growth Inhibition of *Aspergillus fumigatus* in Different Molecular Weight (MW) Chitosan

4. Conclusion

In this study High Molecular Weight Chitosan (HMWC) at 1.5% concentration, Medium Molecular Weight Chitosan (MMWC) and Chitosan synthesized from Crab shell (CSCS) at 2.0% concentration were best for control of *A. fumigatus.* Hence can be utilized as alternatives to synthetic fungicides as the benefits accrued will go a long way to benefit the society immensely at large.

Compliance with ethical standards

Acknowledgments

I acknowledged the leadership of Nigeria Stored products Research Institute and Federal University of Technology for the opportunity to carry out the research

Disclosure of conflict of interest

Authors have no conflict of interest.

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