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DETECTION OF AFLATOXINS PRODUCED BY *ASPERGILLUS* SP. ISOLATED FROM GRAINS

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Abstract:
The present study was planned to assess the aflatoxins contamination by Aspergillus in grains of maize, rice, wheat and groundnut; some detoxification methods were also studied. From the grains fungus Aspergillus was isolated and identified based on morphological and microscopic features. 3 isolates out of 4 were probably identified as belonging to Aspergillus species and identified isolates were screened for aflatoxins production. Screening for aflatoxins production showed that two isolates, isolated from groundnut and maize, were found to produce aflatoxins as these produced intense yellow orange colour on selective media and blue fluorescent ring under UV light. These two isolates were selected for further analysis of aflatoxins by TLC and ELISA. The results showed that the fungi isolated from groundnut produced more quantity of aflatoxins as compared to fungi isolated from maize i.e. the fungi present in groundnut had greater potential for aflatoxins production. Detoxification of aflatoxins, by chemical (sodium bisulfate) and biological (bacteria B. subtilis) methods revealed that both the methods were effective against this aflatoxigenic fungus. The results of this study showed the contamination of aflatoxins in grain samples and gave further evidence of suitability of screening by microbiological method and detection of aflatoxins by TLC and ELISA, which were reliably low-cost approach to determine food and feed biosafety. Further investigations are required for detoxification of aflatoxins by testing health safe chemicals and biological agents. More and more cost-effective and less time consuming, methods should be developed that can give quantitative results accurately at faster rate.

Key words: *Aflatoxins, Biological, Mycotoxins, Quantitative, Detoxification*

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INTRODUCTION

Living in the present time is dangerous, not only because of accidents and environmental hazards, but also because of the foodstuffs that we consume every day. Most of the foods that we eat are contaminated with microorganisms as well as their toxins. Intake of food materials affected by fungi cause the induction of mycotoxicoses (toxic syndrome). This is a disease in which these affected food products produce toxic substances called mycotoxins. Mycotoxins are the secondary metabolites of fungi/ molds [1] and aflatoxins are naturally occurring mycotoxins that are produced by different species of toxigenic fungi (*Aspergillus flavus* and *Aspergillus parasiticus*). Humans can be exposed to aflatoxins by the periodic consumption of contaminated food, contributing to an increase in nutritional deficiencies, immunosuppressant and hepatocellular carcinoma. [2] There are thirteen different types of aflatoxins produced in nature but type B1 is one of the most potent naturally occurring hepatotoxin, teratogen, carcinogen and immuno-suppressive agent effecting both human beings and animals.[3] Environmental factors that favors aflatoxins contamination include high temperature, high relative humidity, high rates of transpiration, reduced water availability, drought stress, nitrogen stress etc. The most important factors are grain moisture content and temperature.[4] The most important factors that help predict the occurrence of aflatoxins in food include weather conditions (temperature and atmospheric humidity), agronomical practices (crop rotation and soil cultivation) and internal factors of the food chain (drying and storage conditions). A comprehensive approach is needed to identify and control risks related to food production system that could present a potential hazard to human health. The emerging risks need to be identified as early as possible in order to take appropriate preventive measures. Thus, the specific risk can be prevented from becoming a danger.[5] Consumption of even extremely small amount of aflatoxins damages various internal organs and can induce development of cancer. Growth of aflatoxins in diet and increase in the rate of this toxin from the critical range (20µg/kg in diet and 0.5µg/kg in milk) cause decrease in fertility and reproductive ability in animals. This is of great concern to the Food and Drug Administration. [6] Removal or inactivation of aflatoxin in food and feedstuffs is a major global concern. The occurrence of fungi and mycotoxins can be controlled by applying a number of preventive measures both before and after harvest, including insect control, good harvesting, drying, and storage practices. If mycotoxins contamination has occurred, the levels of toxins can be reduced by physical, chemical or biological decontamination. Milling, food processing, and regulatory control of toxins to safety levels can also have a positive impact on food safety.[7] In view of mentioned harmful effects of aflatoxins in our food products, the present study was planned to quantitatively analyze aflatoxins in stored grains and detoxification strategies to control these toxins.

MATERIALS AND METHODS

For the isolation of aflatoxins producing fungi grain samples of maize, wheat, rice and groundnut were collected from grain market and storage shops. One gram of each sample (maize, wheat, rice and groundnut) was washed with distilled water, then surface sterilized with sodium hypo chloride solution. The washed samples were inoculated on potato dextrose agar (PDA) plates, incubated for 5 days at 28ºC, examined daily for growth and spore formation. After 5 days of incubation, the different fungal colonies were, sub-cultured on freshly prepared PDA plates and incubated for 5 days. After 5 days, pure fungal colonies were stored in PDA slants and maintained at 4ºC for the identification of fungus isolated from grain samples.

Morphological and Microscopic Characterization of Fungal Isolates

The morphological characteristics of each fungal isolate were analyzed by observing the colour of the colonies and conidia morphology. Microscopic features of fungal isolates were studied by lacto phenol cotton blue (LCB) wet mount method.[8]

Screening of Fungal Isolates for Aflatoxins Production

Screening for aflatoxins production was carried out by initial screening using Aspergillus Flavus Parasiticus Agar (AFPA) medium and then by microbiological method.

Initial Screening by AFPA Medium

AFPA media described by Pitt *et al*. [9] is selective medium used for the rapid screening of aflatoxins producing *Aspergillus* species. The target colonies develop an intense yellow/orange colour on the reverse of the colony, allowing each fungal isolate to be easily differentiated. A loopful of identified fungal isolate was inoculated on AFPA media plates and plates were incubated at 30ºC for 3 days. After 3 days of incubation, the plates were observed under UV light (365 nm) and the development of intense yellow orange colour on the base of colonies indicated the production of aflatoxins. Assante *et al*. [10] showed that the orange yellow colouration was due to the reaction of ferric ions from ferric citrate with aspergillic acid molecules forming a coloured complex.

Microbiological Method of Screening

Microbiological method was reported by Jaimez Ordaz *et al*. [11] for detection of aflatoxins produced by fungus. Fungal isolates (one isolate/ plate) were inoculated on yeast extract sucrose agar (YESA) media and incubated at 28ºC for 3 days. After 3 days of incubation, the aflatoxinogenic fungal isolate formed a beige (white/gold/yellow) ring surrounding the colony which was visible without the need of UV light. It is also possible to visualize the blue fluorescent ring around colonies under UV light.

Analysis of Aflatoxins Produced by Fungal Isolates

Thin layer chromatography (TLC) was suggested as a simple and reliable technique for detection of aflatoxins. [12] Single colony of aflatoxigenic fungi was tranferred to eppendorf containing 500µl chloroform, agitated for 20 minutes at 4000 rpm and after agitation, dried at room temperature. The residue left after drying was dissolved in 10µl chloroform for spotting of the TLC plate. The solvent and spots were allowed to rise until it almost reached the top of the plate. The plates were dried and observed under UV light (360nm). The blue or green fluorescent spots indicated the aflatoxin production. The R_f values of aflatoxins were calculated by knowing the distance travelled by the culture extract and solvent respectively.

$$R_f = \frac{\text{Distance travelled by culture extract spot}}{\text{Distance travelled by solvent}}$$

Quantification of Aflatoxins by ELISA

The enzyme linked immunosorbant assay (ELISA) has been described as analytical method used for the rapid analysis of aflatoxins. This technology is based on the detection of specific proteins found in all aflatoxins using antibodies to identify these proteins. [1] 50µl of sample and 50µl of enzyme conjugate

solution were added in ELISA plate, mixed gently and incubated at room temperature for 30 minutes. After incubation, washing was done with washing buffer 3-4 times. Then, 100µl of enzyme substrate was added into wells, incubated at room temperature for 15 minutes and observed for change in colour. Blue colour indicated the reaction of sample with enzyme substrate solution. 10µl stop buffer was added to stop the enzyme reaction. The intensity of the coloured solution in the wells was measured optically using an ELISA reader at an absorbance of 450nm. The concentration of aflatoxins in each sample was calculated by using the standard aflatoxin curve. [13]

Detoxification Strategies for Control of Aflatoxins

Physical, chemical and biological methods have been investigated in order to prevent the growth of aflatoxigenic fungi, to reduce or eliminate the toxin levels, to degrade or detoxify the toxins in foods and feeds. These strategies involved the use of certain chemicals or biological species or physical treatments (heat) to inhibit or control aflatoxins in food products. [14] In this study, chemical treatment and biological methods were adopted to inhibit the growth of aflatoxins producing fungi. For these methods, 10 ml of potato dextrose broth (PDB) was inoculated with isolated fungal culture and incubated for 48 hours at 28ºC in incubator.

For chemical treatment, 100µl of fungal spore suspension from test tube containing PDB was spread on the PDA plates. Then, wells were made in the plate and 20µl of sodium sulphite (1gm/10ml distilled water) solution was added. The plates were incubated at 28ºC for 2 days and observed for the zone of inhibition around the wells. *Bacillus subtilis* (MTCC 281) was tested for the inhibition of aflatoxigenic fungi in biological treatment.100µl of the fungal spore suspension from test tube containing PDB was spread on the PDA plates. Then, the wells were made in the plate, 20µl of bacterial suspension was added in the well and zone of inhibition around the well was observed after incubation of 2 days at 28ºC.

RESULTS AND DISCUSSION

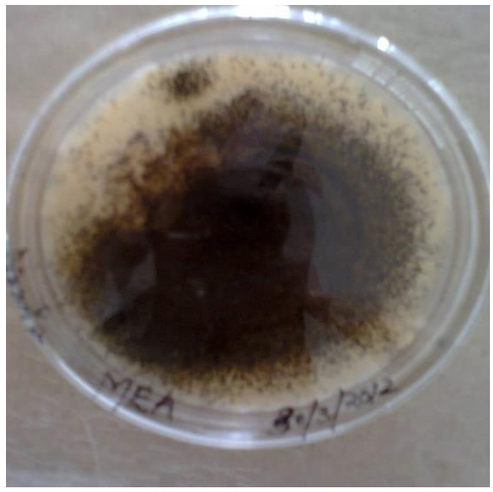
Isolation and Identification of Fungi

Four filamentous fungi were isolated from grain samples. The fungal isolates were identified based on growth, colony colour, conidia morphology and microscopic features (Fig.1).

Fungal Isolate 1

Fungal Isolate 2

Fungal Isolate 3



Fungal Isolate 4

Fig1: Fungal Growth after Sub-culturing on PDA Plates

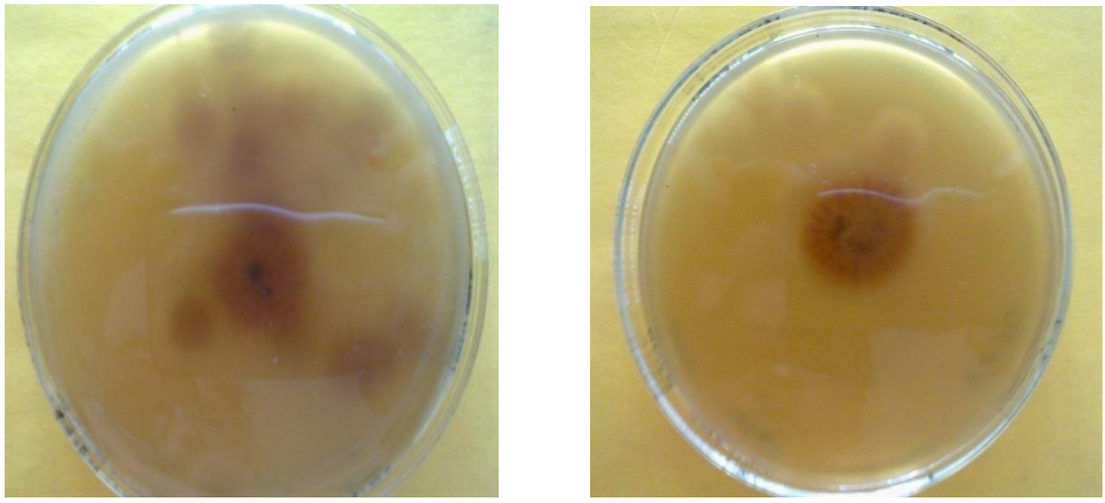


Fig 2: Orange Colour Production on the Reverse Side of Plate by Fungal Isolate 1 and 3 on AFPA Plates

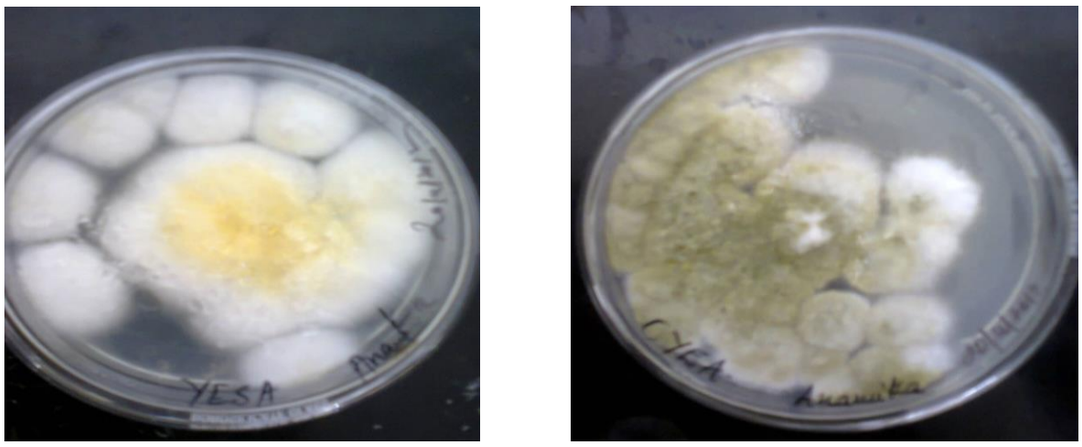


Fig 3: Beige Ring around Fungal Isolate 1 and 3 on YESA Plates

On the basis of morphological and microscopic observation, it was found that fungal isolate 1, 3 and 4 belong to *Aspergillus* species and fungal isolate 2 to *Rhizopus* species. Three fungal isolates belonging to *Aspergillus* species (probable identity) were further screened for production of aflatoxins.

Screening of Fungal Isolates for Aflatoxins Production

Two fungal isolates (1 and 3) out of tested three produced a bright intense orange colour on the reverse side of plate indicating aflatoxins production (Fig.2). When observed by microbiological method, beige (gold/yellow) ring was observed around the isolate 1 and 3 but no colouration was observed around the isolate 4 (Fig.3).

When observed under UV light, fungal isolate 1 (isolated from maize) showed purplish ring while fungal isolate 3 (isolated from groundnut) produced blue fluorescent ring indicating that aflatoxins production by fungal isolate 3 was more than fungal isolate 1. This result of present study was found to be comparable with the results of study conducted by Ordaz *et al*. [11] which suggested that the aflatoxins producing isolates are more prevalent in nuts as compared to other samples.

Analysis of Aflatoxins Produced by Fungal Isolates

TLC is a simple and easy technique to detect the aflatoxins. The retention factors (R_f) of each identified fungal isolate was determined by observing the distance travelled by the fungal isolate and solvent (Table 1; Fig. 4).

Table 1: Retention Factor (R_f) Value for Aflatoxins

Fungal Isolate	Distance Travelled by Isolate (cm)	Distance Travelled by Solvent (cm)	Retention Factor (R_f) Value	Type of Fluorescence	Aflatoxin
1	1.9	4.0	0.49	Blue	B
3	3.0	5.2	0.59	Greenish-yellow	G

Fig 4: TLC Plate Showing Development of Chromatogram

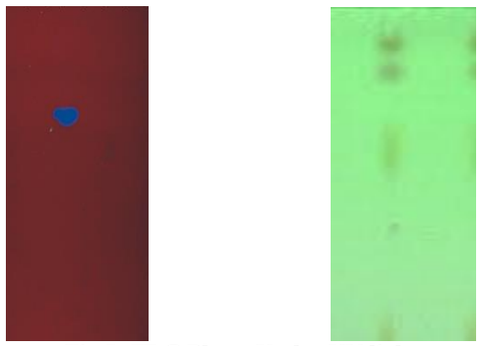


Fig 5: TLC Plates Under UV light

R_f value of fungal isolate 3 was found to be more (0.59) than fungal isolate 1 (0.49) indicating that fungal isolate 3 produced more aflatoxins as compared to fungal isolate 1. Blue colouration under UV light produced by fungal isolate 1 showed that the aflatoxins produced by this isolate belong to B type and greenish-yellow colouration under UV light by fungal isolate 3 corresponds to G type aflatoxins (Table 1 & Fig. 5). R_f values calculated in present study were found to be in accordance with previous study conducted by Amadi and Adeniyi [15], they reported R_f value 0.44 and 0.56 for *A. flavus* and *A. paraciticus* respectively.

Quantification of Aflatoxins by ELISA Method

This technique was used to assess the level of aflatoxins in the grain samples analyzed in this study. It is cost and time effective method to monitor the aflatoxins contamination in the large number of samples. The optical density for each sample was measured using ELISA reader. Aflatoxins concentration for each sample was calculated using standard aflatoxins curve. The concentration of aflatoxins was found to be maximum in groundnut (7.85ng/g) followed by rice (6.27ng/g), maize (5.01ng/g) and wheat (2.43ng/g) (Fig.6).

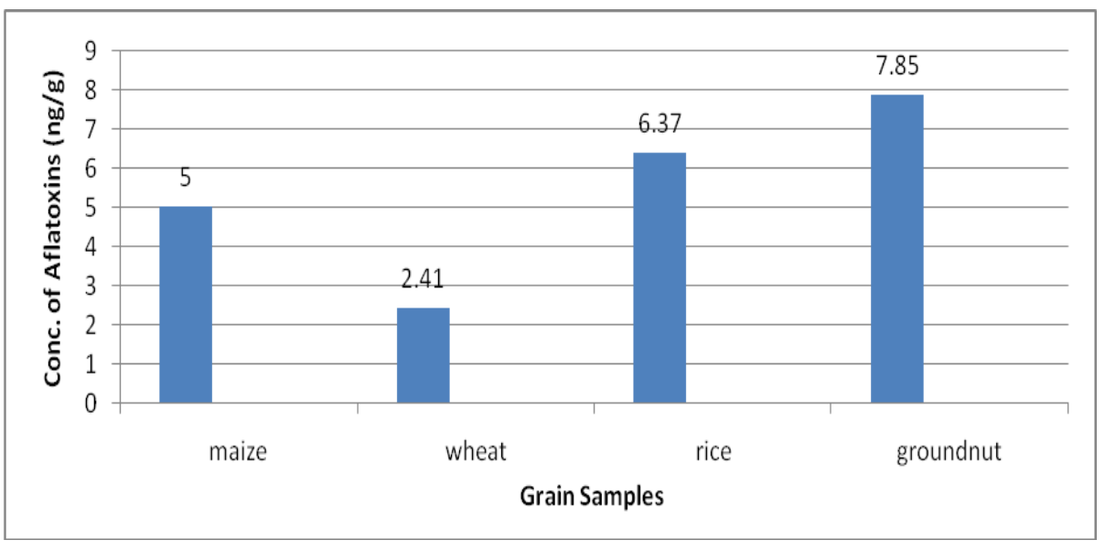


Fig 6: Aflatoxins Concentration (ng/g) in Different Grains

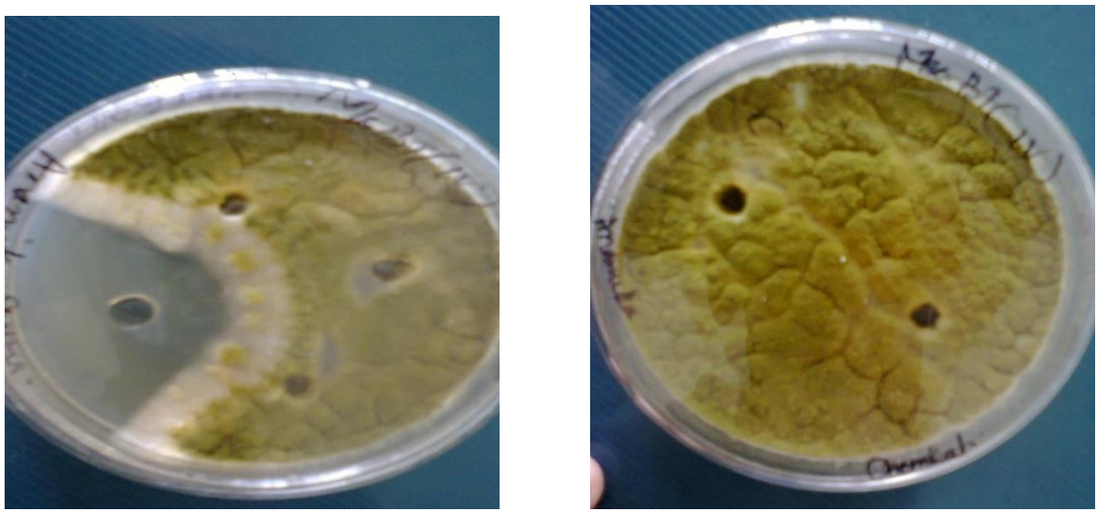


Fig 7: Zone of Inhibition Produced by Fungal Isolate 1 and 3 on PDA Plates

From the results of TLC and ELISA, it can be concluded that fungi isolated from groundnut produced more aflatoxins as compared to other grain sample indicating that groundnut was found to be more susceptible to aflatoxin contamination. Earlier study by Ayejuyo *et al*. [16] also found the highest level of aflatoxins in the groundnut sauces although other commodities were also at a risk of aflatoxins. The aflatoxin contents of groundnut ranged from 6.25ng/g to 7.80ng/g that is very near to aflatoxins content of groundnut in the present investigation.

Detoxification Strategies for Control of Aflatoxins

The aflatoxin producing fungal isolates (1 and 3) were inhibited by the use of sodium bisulfite. The zone of inhibition was observed for each fungal isolate around the well after the incubation of 3 days.

Results of chemical detoxification showed that fungal isolate 1 was more susceptibility to sodium bisulfite (zone of inhibition 12mm) as compared to fungi isolated from groundnut (isolate 3) with zone of inhibition 8mm indicating that this chemical method had potential to inhibit the growth of fungus in grains responsible for aflatoxins (Fig.7). Earlier studies showed that treatment of *Aspergillus flavus* inoculated groundnut cake with 1% sodium bisulfite at 10% moisture completely inhibited mold growth and aflatoxins production at room temperature. Moisture, sodium bisulphite level and temperature had significant effects on aflatoxins degradation (Abbas *et al*., 2006; Hagler *et al.*, 1982). [17,18]

B. subtilis showed greater inhibitory effect on fungal isolate 1 as compared to isolate 3 evidenced from inhibition zones. The zone of inhibition of fungal isolate 1 isolated from maize was found to be larger as compared to isolate 3 isolated from groundnut. (Fig. 8).

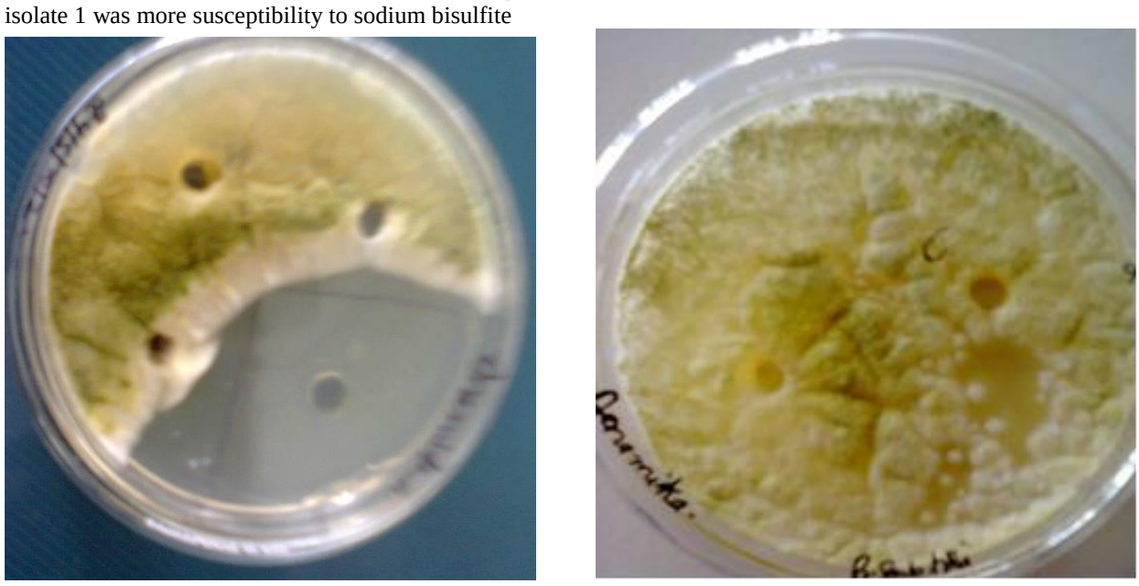


Fig 8: Zone of Inhibition Produced by Fungal Isolate 1 and 3 on PDA Plates

Hai [19] suggested that *Bacillus subtilis* had potential to reduce the aflatxoxin poduction by fungus. Research on the use of *Bacillus* strains and their metabolites to reduce the aflatoxins is still going on. Therefore, the potential use of these microbial metabolites to control mycotoxigenic fungi should be thoroughly investigated since many chemical fungicides are being taken out from the commercial market.

CONCLUSIONS

In conclusion, this study showed the presence of aflatoxins in grains of maize and groundnut. Chemical and biological methods had been investigated in order to prevent the growth of aflatoxigenic fungi and these methods were found effective to control the growth of aflatoxins producing fungi. These methods can be used to prevent aflatoxins contamination. Because of the *B. subtilis* inhibitory activity against the aflatoxigenic fungi, the bacteria may be useful as potential biocontrol agents against aflatoxigenic fungi during food storage. Mycotoxins pose a serious threat to animal and human health and efforts continue to be devoted worldwide, to prevent or eliminate them. Vast work for analysis of aflatoxins will serve the purpose of alerting the consumers on the dangers of consuming poorly stored grains. Further research is needed to explore other means of preventing mould infection and aflatoxins contamination in the field through the use of biological control agents that are pathogenic to *Aspergillus flavus*, such as yeast or harmless bacteria. Genetically engineered antibody to toxin production should be explored for the prevention of toxins produced by mould. Finally, it can be concluded that economical methods of removal of aflatoxins from food and feed may be used on large scale to minimize economic loss due to aflatoxins contamination and to improve animal and human health condition.

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