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Research paper

Determination of Mycotoxins in Food Condiments by the ELISA method

Aisha Ahmed, Hafsa Nazir, Rimal Rafiq, Sara Riaz, Qandeel Laraib*

Department of Biosciences, Mohammad Ali Jinnah University, Karachi, Pakistan.

*Corresponding Author: Dr. Qandeel Laraib (qandeel.laraib@jinnah.edu).

ABSTRACT

Fungi being an intrinsic constituent of the earth cause contamination in many foodstuffs including food condiments among these fungi are the Aflatoxigenic fungi, Aflatoxins are considered the most poisonous mycotoxin that is produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Exposure to Aflatoxins can lead to aflatoxicosis which can be life-threatening as it could damage the liver, Aflatoxins are found to be mutagenic as well as carcinogen. Aflatoxin is so harmful that WHO has grouped it into a group 1 carcinogen. Considering these aspects, this study was designed to investigate the presence of Aflatoxigenic fungi food condiments sold in Karachi, Pakistan. Many samples (local and branded) of many different categories of food condiments including Ketchup, Spices, and Vinegar are collected. After serial dilutions, the samples were spread on Sabouraud's dextrose agar and incubated for 7 days at room temperature. The fungal colonies which appeared were then identified based on microscopic (using lacto phenol cotton blue stain) and macroscopic features, and then preserved using the spore suspension method.

The preserved fungal isolates were screened for the presence of Aflatoxigenic fungal strains using the cultural technique (Ammonium hydroxide vapor test) and further quantitative analysis was carried out by ELISA technique and the results contained aflatoxin levels of 4.437 ppb in Vinegar, 2.982 ppb in ketchup, and 24.053 ppb in Black pepper. Further, the biocontrol of aflatoxin was designed using *Calocybe indica* by providing different natural conditions like carbon source, temperature, and pH.

KEYWORDS Aflatoxin, Food Condiments, Sabouraud's dextrose agar, Lactophenol cotton blue stain, Ammonium Hydroxide test.

INTRODUCTION

Food condiments are special preparations that are added to our food after cooking and most probably before serving which enhances both flavor and aroma of the food. These food condiments include ketchup, spices, herbs, sauces, spreads, pickles, etc. Different types of condiments are routinely used in different parts of the world and are

widely consumed by populations of all socioeconomic classes. Consequently, they could serve as food vehicles for micronutrient fortification to combat micronutrient deficiencies in many countries, especially in South America and Asia [1]. Like other food products, food condiments' acidic pH and moisture content facilitates the growth of different fungi in them.

Many fungi that grow in food products are known to cause illness in animals, plants, and humans. Among 1.5 million estimated fungal species, 300 of them are able to cause severe health conditions and invasive infections also

termed systematic mycoses [2]. Contaminated food can be harmful to consumers due to the production of secondary metabolites such as toxins in food products. Some pathogenic fungi that grow and contaminate food are Aspergillus, Alternaria, Fusarium, Candida, and Mucormycetes [3].

Aflatoxins are considered the most poisonous mycotoxin produced by the *Aspergillus* species. These species are ubiquitous. The high ecological, biological, and metabolic diversity of Aspergillus species led to the exploration of secondary metabolites among these species [4]. *Aspergillus flavus* and *Aspergillus parasiticus* are major species that are known to produce aflatoxins [5]. Aflatoxigenic fungi affect the different crops which include corn, wheat, rice and sorghum, soybean, peanut, cotton and sunflower seeds, chili peppers, turmeric, coriander, ginger, black pepper, and pistachio, coconut, walnut, Brazil nut, almond and can also be found in the milk of those animals that are fed with aflatoxigenic fungal contaminated feed. The limit of aflatoxin presence in food is 20 ppb per 5ml/gm and is changed respectively according to the food or crop. Exposure to aflatoxins can lead to aflatoxicosis and this can be life-threatening because it can damage the liver. Aflatoxins have also been shown to be mutagenic; they can damage DNA and cause cancer in animals. There is also evidence that they can cause liver cancer in humans.

Chemically, aflatoxins (AFTs) are difuranocoumarin derivatives. A bifuran group is attached to one side of the coumarin nucleus, while a pentanone ring is attached to the other side in the AFTs-B series, and a six-membered lactone ring is attached in the AFTs-G series [6]. Around 18 different types of aflatoxins have been identified and among them, six types are considered important and are named AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2. These aflatoxins are separated from each other on the basis of molecular differences [7].

MATERIALS AND METHODS

Various samples of four different categories of locally available food condiments of different brands i.e., ketchup, spices (chat masala, black pepper), vinegar, and sauces (soy sauces, chili sauce, chipotle sauce) were collected by convenient sampling method in clean autoclaved Eppendorf tubes.

An appropriate amount of Sabouraud's Dextrose Agar (SDA) and agar were suspended in distilled water and boiled for about a minute or two to completely dissolve the media. The media was then autoclaved and cooled for up to 45 to 50°C and further poured into Petri dishes.

The fungal isolates were then identified by macroscopic and microscopic characteristics.

For the macroscopic identification, the color, texture, and reverse of colonies were examined.

For microscopic identification, the tease mount technique was used. A drop of lactophenol cotton blue dye (LPCB) was placed on a clean grease-free glass slide. A small portion of the colony was transferred onto the drop of LPCB and using a sterile fungal needle the growth was gently teased. A cover slip was placed and finally examined under the microscope in low power 10X and high power 40X magnification.

The spore suspension was made for the preservation of fungal cultures. The spore suspension was made by adding 0.05% tween 80 into the autoclaved distilled water in a flask to make a volume up to 100ml. The flask was then autoclaved. After the autoclave, pure fungal culture was scratched and added to the flask. This process was repeated about 15 to 20 times.

Tween 80 was added to the water to avoid the clumping of fungal particles. Then the flask

was placed onto the shaker for 30 minutes at 150 rpm. After twenty minutes, all the pieces of culture were mixed in the suspension. The spore suspension was preserved for around two months at room temperature.

The Aflatoxin-producing strains of *Aspergillus flavus* and *Aspergillus parasiticus* were detected using a cultural method known as the ammonium hydroxide vapor test. A single fungal colony was cultivated in the middle of a petri dish using Sabouraud's dextrose agar. A drop or two of pure ammonium hydroxide was then poured inside the lid of the plate after it was turned upside down. While the reverse of non-producing colonies underwent no color change, the reverse of Aflatoxin-producing colonies quickly turned plum red.

Further screening of colonies positive for AHT was done using enzyme-linked immune sorbent assay (ELISA KIT BZERO H2AFLA) for confirmation and quantification of aflatoxin production. The sample weighing 5gm was added into 25ml of extraction solvent. It was further mixed vigorously for 2-3 minutes then the mixture was filtered by using filter paper. Next 50µl of the sample and control were added to the mixing well and incubated for 5 minutes. After 5 minutes, the mixtures were transferred from mixing wells to coated wells having anti-aflatoxin antibodies. Wells were washed after 5 minutes by wash buffer. Then developing solution was added and incubated for 5 minutes. After 5 minutes, stop solution was added and the plate was kept in ELISA reader.

Biocontrol against Aflatoxin Positive *Aspergillus flavus* was designed using beneficial fungal specie known as *Calocybe indica* using the following steps:

Preparation of Cell-Free Filtrate

Calocybe indica (white mushroom) was grown in Sabouraud's dextrose broth (SDB) for 7 days then its cells were harvested and lysed by filtering followed by centrifugation of the cells obtaining its cells free filtrate (CFF) at 150rpm for 10minutes.

***Calocybe indica* Against Aflatoxin Producing Aspergillus Species on SDA Media**

The lawn of Aflatoxin positive specie of Aspergillus was prepared on Sabouraud's dextrose agar media plates. Wells were made using a borer. Around, 100 µL of cell-free filtrate (CFF) of *Calocybe indica* was loaded in one well and 100 µL of an anti-fungal agent (Nystatin as a positive control) was loaded in the other well.

The plate was then incubated for around 5-7 days. After the incubation period, zones of growth inhibition were recorded.

Optimization of Bioactive Metabolite Production by *Calocybe Indica* Against Aflatoxin Positive Specie

The one factor/variable at a time optimization (OFAT/OVAT) was carried out and the factors considered were: Carbon source, temperature, and pH. Fungal minimal media was prepared for carrying out the process of optimization.

Screening of Cell-Free Filtrate of *Calocybe*

Effect of Different Carbon Sources

In order to elevate the inhibitory effect of the bio-active metabolite of *Calocybe indica* against *Aspergillus flavus* different carbon sources were used that are wheat bran, rice bran, and starch. In three different flasks, 1% of each of the carbon sources along with the fungal minimal media was added followed by the inoculation of *Calocybe indica* fungi. The flasks were then incubated for 7 days. A lawn of *Aspergillus flavus* was prepared on SDA media and wells were made using a borer. Also, Cell free filtrates of all three carbon sources were made. In wells each of the CFF, antifungal agent (Nystatin, positive control) and negative control (having media no fungi) were loaded in the wells. The plate was then incubated for 7 days and later the carbon source showing the largest zone of growth inhibition was selected to proceed with.

Effect of Different Temperatures

In three different flasks, the fungal minimal media with an optimized carbon source was added along with the inoculation of *Calocybe indica*. Each of the three flasks was then incubated at three different temperatures: 20°C, 40°C, and 60°C. A lawn of *Aspergillus flavus* on SDA media, wells were made using a borer. Also, Cell-free filtrates of minimal media having optimized carbon source and the fungi were prepared.

In the wells, each of the CFF, antifungal agent (Nystatin, positive control), and control (having media no fungi) were loaded in the wells. The plate was then incubated for 7 days and the temperature at which the largest zone of growth inhibition was observed was selected for further processing.

Effect of Different pH Values

Different pH values 5, 7, and 9 were considered for further optimization. In three different flasks, the fungal minimal media with optimized carbon source and the optimized temperature was added along with the inoculation of *Calocybe indica* followed by the pH adjustment at desired values by preparing 0.1 N NaOH or 0.1 N HCl buffer. The flasks were then incubated. Again, on an SDA plate lawn of Aflatoxin positive specie of *Aspergillus flavus* was prepared; wells were made using a borer. Also, Cell-free filtrates of minimal media having optimized carbon source, temperature, and fungi were obtained.

In wells, each of the CFF, an antifungal agent (Nystatin, positive control), and negative control were loaded in the wells. The plate was then incubated for 7 days and the well with the largest zone of inhibition was selected.

RESULTS AND DISCUSSION

Twenty-three different samples of food condiments (Ketchup, Spices, Vinegar, and Sauces) were collected and proceeded for the isolation of fungi.

Eight different types of fungal isolates were isolated from various locally available processed Ketchups and identified which were *A. flavus, A. fumigatus, A. niger, A. versicolor, A. terreus, Apophysomyces elegans, Rhizomucar spp, Syncephalastrum racemosum*. *Asp flavus* was also identified.

Four different types of fungi namely *A. flavus, A. fumigatus, A. niger, A. versicolor* were identified from chat masala and black pepper samples. *A. flavus* was isolated.

Five different brands of Vinegar were tested and three different types of fungal isolates

were identified as *A. flavus*, *A. niger*, and *A.versicolor*. *A. flavus.* Five different brands

of soy sauces, chili sauce, and chipotle sauce were also screened for fungi and only *A. niger* was isolated (Figure 1).

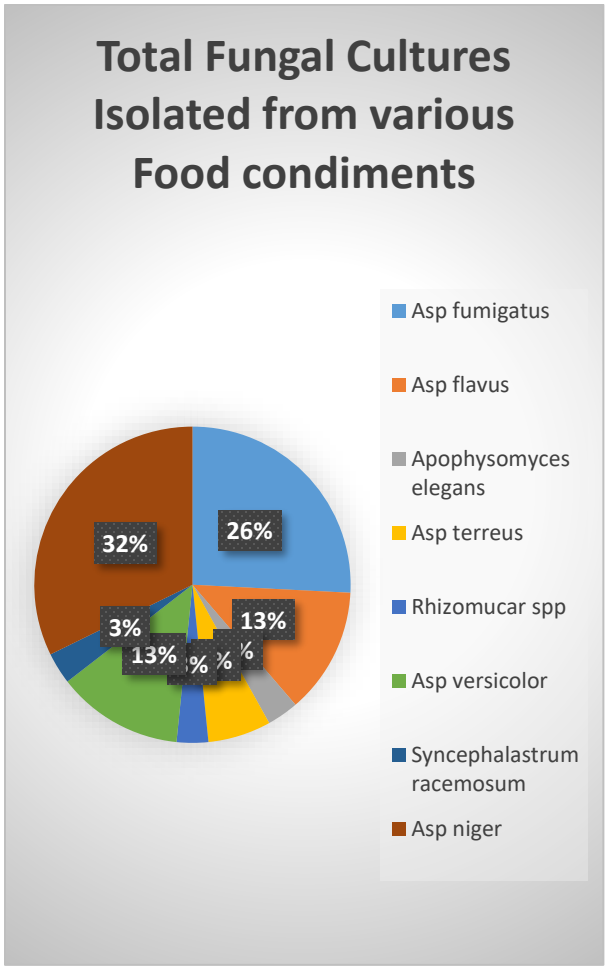

Figure 1: Fungi isolated from various food condiments

Screening of *Aspergillus flavus* and *Aspergillus parasiticus* for the Aflatoxin production has been done through the Ammonium Hydroxide Vapor Test. *A. flavus* isolated from the Black Pepper sample showed a plum red color as an indication of aflatoxin-positive culture (Figure 2).

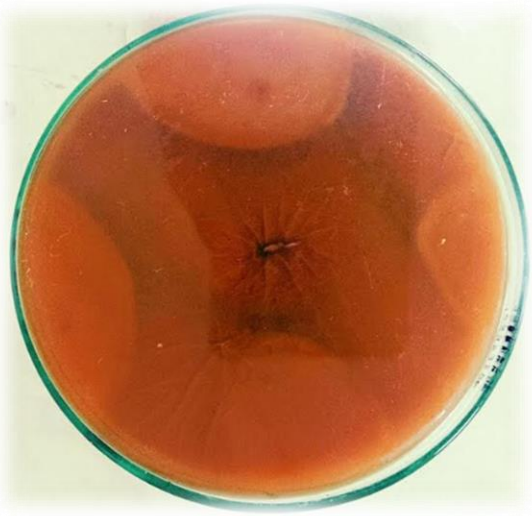

Figure 2: *A. flavus* isolated from the Black Pepper sample was screened as positive through Ammonium Hydroxide Test.

Quantitative screening of the condiment sample was further carried out using ELISA technique. The standard limit of aflatoxin in food or crops is 20 ppb per 5ml/gm sample.

The aflatoxin levels were estimated as 4.437 ppb in Vinegar, 2.982 ppb in ketchup, and 24.053 ppb in Black pepper.The optimization of various environmental and nutritional factors for the production of bioactive metabolite by *Calocybe indica* was carried out. *Calocybe indica* produced a clear zone of growth inhibition against Aflatoxin-positive *A.flavus* (47.8 mm) when *C. indica* was cultivated in the presence of wheat bran as a carbon source (Figure 3).

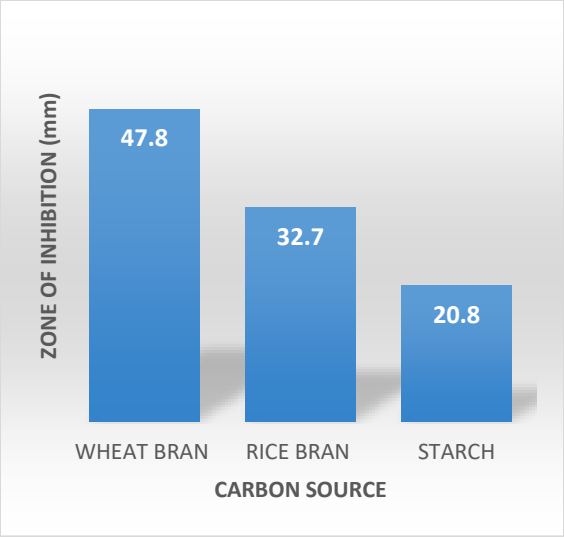

Figure 3: Effect of Different Carbon Sources on Growth Inhibition of Aflatoxin-positive *A.flavus*

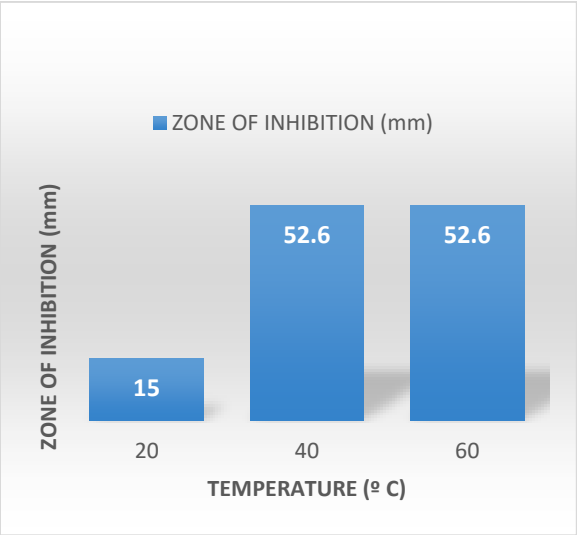

Figure 4: Effect of Temperatures on Growth Inhibition of Aflatoxin-positive *A.flavus*

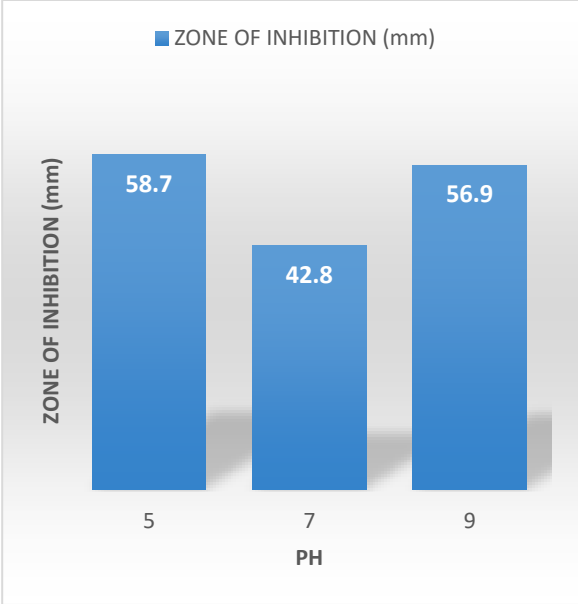

Figure 5: Effect of pH on Growth Inhibition of Aflatoxin-positive *A.flavus*

CONCLUSION

The aflatoxin-producing fungi are widely spread in the environment and can cause severe contamination in food supplies which results in health hazards and even death. Food condiments are used worldwide to enhance food taste and flavor, and some are used without any pretreatment before consumption. Various samples of commonly used food condiments of renowned brands as well as from local market were collected and processed for the detection of aflatoxigenic fungi. Aflatoxigenic fungi Aspergillus flavus was isolated. Also, a few other fungal species were isolated from these samples. Out of 23 samples, 13% samples were found to be aflatoxin positive. According to the results of ELISA, the aflatoxins were detected at concentrations ranging from 2 ppb to 24 ppb. Among all samples, only one sample exceeded the maximum limits of 20ppb as given in the protocols and literature. There is a need for continuous monitoring of

Aflatoxins in food condiments. Biocontrol against aflatoxigenic fungi was developed by using a mushroom-forming fungi *Calocybe indica*. This fungus showed satisfying results in controlling the aflatoxigenic *Aspergillus flavus.* Therefore, the present study concluded that the Aflatoxigenic fungi can be controlled by biological control approaches and further studies should be carried out at the molecular level to study which bioactive compounds are produced against toxigenic fungi to control aflatoxin contamination.

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