CO-CULTURE EFFECT OF TOXIGENIC AND ATOXIGENIC ISOLATE OF *ASPERGILLUS FLAVUS* ON AFLATOXIN BIOSYNTHESIS

Project report submitted in partial fulfilment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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CERTIFICATE

I hereby declare that the work presented in this report entitled **"Co-culture effect of Toxigenic and Atoxigenic isolate of Aspergillus Flavus on Aflatoxin Biosynthesis"** in partial fulfillment of the requirements for the award of the degree of **Bachelor of Technology** in **Biotechnology** submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out overa period from August 2022 to May 2023 under the supervision of **Dr. Jata Shankar** (Professor, Department of Biotechnology and Bioinformatics).

I also authenticate that I have carried out the above-mentioned project work under the proficiency stream.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is true to the best of my knowledge.

(Supervisor Signature)

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LIST OF ABBREVATIONS

1	DNA	Deoxyribonucleic acid
2	RNA	Ribonucleic acid
3	*A.Flavus*	*Aspergillus flavus*
4	PDA	Potato dextro agar
5	PDB	Potato dextro broth
6	*aflR*	gene encoding a Zn(II) 2 Cys 6 DNA-binding domain
7	*aflJ*	Gene cluster
8	ORF	Open Reading frame
9	Kb	Kilobyte
10	AFB1	Aflatoxin B1
11	AFG1	Aflatoxin G1
12	AFM1	Aflatoxin M1
13	PPB	Parts per billion
14	Ml	Microliter
15	HCC	Hepatocellular carcinoma
16	UV	Ultraviolet

ABSTRACT

Aflatoxin contamination of food intake is an issue both in developing and developed nations, particularly when there is poor control over aflatoxin levels in food. AFBs, specifically AFB1, are bio-transformed in the body into a variety of metabolites, including the active AFB1-exo- 8,9-epoxide (AFBO). The AFB, AFBO, and other metabolites interact with a wide range of biomolecules in the body, including nucleic acids like DNA and RNA as well as the various metabolic pathways like protein synthesis, the glycolytic pathway, and the electron transport chain that are all involved in ATP synthesis in body cells. AFB-DNA adducts are created when the AFB interacts with DNA, breaking the DNA. Aflatoxin contamination of agricultural products and processed foods is a known problem, but there are other mycotoxins as well. *Aspergillus* and *Penicillium* fungus lead the production of these substances. The primary foods of the majority of developing nations, cereals and their products, are particularly susceptible to infection by aflatoxigenic fungus. Accordingly, this review looks at the various types of aflatoxigenic fungi and toxins, their presence in food products, their negative effects, the resulting economic losses, regulation, including the permissible limits set by various national and international agencies, and how their effects can be reduced or completely avoided. Due to their lack of resources, developing nations require the financial and technical assistance of their developed counterparts and international organizations in order to start educational, scientific, and other endeavors and ultimately reduce pollution in their goods.

CHAPTER 1

1.1 Introduction

Certain strains of the fungus Aspergillus, particularly *Aspergillus flavus* and *Aspergillus parasiticus*, create a kind of mycotoxin known as aflatoxin. When particular conditions, such as high temperatures and high humidity, are met, such as when they are, these fungus can grow on a range of crops, including peanuts, corn, cottonseed, and tree nuts. As one of the most potent naturally occurring carcinogens, aflatoxin can harm both humans and animals by causing liver cancer and other health issues.

Pre-harvest, harvest, and post-harvest storage are only a few production phases when aflatoxins can contaminate food and feed. Aflatoxin contamination must be controlled by managing crops and storage facilities properly and by routinely checking for aflatoxin levels in foods and feed products. Using biological control agents, spraying fungicides, and optimizing storage conditions including lowering moisture and temperature are some ways to reduce aflatoxin infestation. Aflatoxin exposure can be decreased for consumers by avoiding moldy or discolored food products, handling and storing food safely, and adhering to appropriate food safety procedures. To protect the public's health and safety, several nations and international organizations have created regulations and recommendations for aflatoxin levels in food and feed.

Aspergillus flavus typically grows in soil and decomposing organic waste. Aflatoxin, a carcinogenic mycotoxin produced by it, is known to infect food crops like peanuts, maize, and cottonseed. Since it has been connected to cancer, liver damage, and the weakening of the immune system, aflatoxin contamination poses major health concerns to both people and animals if consumed. It is also recognized that *A. flavus* may infect humans and cause a variety of illnesses, especially in individuals with compromised immune systems such those who have HIV/AIDS, leukemia, or are receiving chemotherapy. Lung, sinus, skin, and other organ infections are all possible, and they can be fatal if untreated. *A. flavus* can be prevented and controlled by taking steps like handling and storing food crops properly, as well as using antifungal medications on high-risk persons. *A. flavus* is the most commonly connected species with aflatoxin contamination of agricultural crops. [1]. The soil-borne pathogen *A.flavus* is more frequently seen on maize, cotton, trees, and ground nuts than on rice in agricultural areas of temperate and tropical countries [2]. *A. flavus* populations are highly diverse, and it is unknown how stable they are in the soil and

on the plant. Fermentation of rice and soybeans typically uses *A. oryzae*, an atoxigenic relative of *A. flavus*. strains that produce toxins and do not produce toxins, different spore types, and strains that react differently to light, The broad collection of strains collectively referred to as *A. flavus* includes strains belonging to distinct vegetative compatibility groups (VCGs), as well as strains with varying abilities to colonise live plant tissue. *A. flavus* is a fungus that mostly lives in soil and is classified as a saprophyte; but, due to its opportunistic nature, it can easily colonise most environments provided there is a rich supply of carbon and nitrogen. It would appear that the variation of *A. flavus* is an evolutionary response to its widespread distribution. Although its main method of reproduction is asexual sporulation, *A. flavus* is also capable of producing sclerotia, which are stiff masses of dehydrated and melanized mycelia that can endure unfavourable climatic and nutritional conditions [3].

Just four species of Aspergillus generate aflatoxins, of which *A. flavus* and *A. parasiticus* are agriculturally significant. Several Aspergilli species and many other non-aflatoxigenic genera of fungus appear to share a portion of the polyketide synthesis pathway that results in aflatoxin. For instance, *A.nidulans* possesses the whole route for the manufacture of aflatoxin, with the exception of the stage when sterigmatocystin, a substance that is equally carcinogenic, is transformed into aflatoxin [4].

Foods often include aflatoxins, especially in the staple diets of many developing nations including India. Several mycotoxins can be produced when fungus infect crops during the processes of harvesting, storing, and shipping them. Aflatoxin is one of the mycotoxins that some fungus makes as secondary metabolites. A number of fungi, including *Aspergillus, Penicillium, Fusarium,* and *Alternaria*, have the ability to manufacture aflatoxins; however, *A. flavus* and *A. parasiticus* are responsible for the production of the most lethal forms of aflatoxins. Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), Aflatoxin M1 (AFM1), and Aflatoxin M2 (AFM2) are the six main forms of aflatoxins. Food crops or their derivatives include B1, B2, G1, and G2 whereas M1 (B1 metabolite) and M2 are found in animal byproducts such dairy products [3]. While *A. flavus* creates Aflatoxin B1 and B2, *Aflatoxin parasiticus* creates Aflatoxin G1 and G2.

They are the most frequent cause of contamination in nuts (almond, pistachio, and walnut), oilseeds (peanut, soybean, sunflower, and cotton), spices (chilli, black pepper), and cereals (maize, rice, and wheat). The fungus *A. flavus* has a green colour and the capacity to multiply under a

variety of difficult conditions. These fungi were able to enterbecause to the damage done by nematodes and insects. The introduction of fungi into crops affects the ability of agricultural plants to defend themselves from fungal attack and contaminates the crop seeds, which in turn promote the formation of aflatoxin [3]. According todata, there are known regulations in 77 countries that regulate the amount of mycotoxin, with 48 having precise regulatory standards for the overall amount of aflatoxins in foods and 21 for aflatoxins in feeds. The FDA has established guidelines for aflatoxin M1- 0.5 ppb and 20 ppb of total aflatoxins for the selling of milk in interstate food and feed commerce [4].

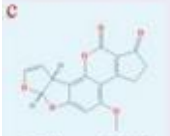
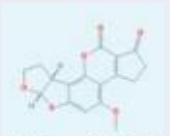
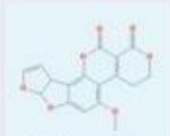
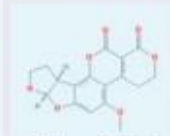
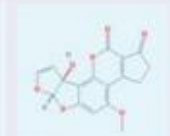
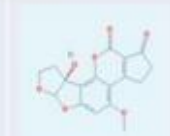
a	AFB1	AFB2	AFG1	AFG2	AFM1	AFM2
b	$C_{17}H_{12}O_6$	$C_{17}H_{14}O_6$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$
c	 PubChem- 186907	 PubChem- 2724360	 PubChem- 14421	 PubChem- 2724362	 PubChem- 15558498	 PubChem- 146159520
d	312.28	314.29	328.28	330.29	328.28	33.29
e	268-296	286-289	244-246	237-240	299	293

Figure 1 – Properties of aflatoxin: a) type, b) chemical formula, c) chemical structure, d) molecular weight(g/mol), and e) melting point.

Adapted from- A. Bansal, M. Sharma, A. Pandey, and J. Shankar, "Aflatoxins: Occurrence, Biosynthesis Pathway, Management, and Impact on Health," in *Fungal Resources for Sustainable Economy: Current Status and Future Perspectives*, 2023, pp. 565–594.

Animals and people who consume aflatoxins are exposed to potent carcinogens. Since its discovery more than 40 years ago, aflatoxin toxicity has been documented in experimental animals and humans to be immunosuppressive, mutagenic, teratogenic, and hepatocarcinogenic. Many physical and chemical elements can affect how bacteria thrive in an artificial media. Nutritional material made specifically for the growth of microorganisms in a lab is known as culture media. The amount of nutrients in a culture medium has a big effect on how quickly bacteria grow. The components of Potato Dextrose Agar (PDA), which is frequently used in laboratories for the isolation and growth of several fungi, are well known. Nevertheless, the high cost and lack of culture medium in underdeveloped nations impede microbiological research. As a result, several researchers have focused in recent years on screening alternate culture medium using inexpensive locally accessible materials. Even though they were able to grow several fungi on the alternative media, they always needed to add agar as a solidifying agent. The price of agar adds to the stress.

The objective of the present research was to find out if four locally accessible materials might provide a nutritive and self-solidifying substrate for the growth of several kinds of fungi [5]. Amylases, important enzymes utilised in the starch processing industries to hydrolyze polysaccharides like starch into simple sugar components, break down the 1-4 linkage of starch. This enzyme is frequently used in the starch liquefaction, sugar, paper, food, and pharmaceutical sectors. Enzymes are among the most important components obtained from microbial sources for human needs.

Due to the newly available prospect of using microbes as biotechnological sources of industrially significant enzymes, interest in the study of extracellular enzymatic activity in diverse microorganisms has recently increased. A family of enzymes known as amylases has been discovered in a variety of microbes, including fungus. Because of their widespread distribution and mild dietary needs, *Rhizopus spp.* and *Aspergillus spp.* have received the most attention in studies on fungal amylase, particularly in underdeveloped nations. *A. niger* strains have been shown to create 19 different kinds of enzymes, although as many as 28 different microbial cultures have also been found to produce the enzyme - amylase. So, choosing a strain that is appropriate for the task at hand depends on a variety of variables, including the kind of substrate and the surrounding environment. For fungi to grow best, growth conditions must be optimized. Fungal strains may have varied growth requirements, despite the fact that cultures of the same species and genus typically thrive under the same conditions [6]. The growth responses of fungi vary from strain to strain even when they are grown under the identical conditions. Since fungi can grow in a wide range of pH conditions, they must be able to modify gene expression to the unique pH of their developing environment. The requirements for pH vary across various filamentous fungi. Most common fungi flourish in the pH range of 3 to 7, while others, like *Penicillium funicilosum, A.niger,* and *Moniliella acetoabutans,* may grow at pH 2 and below. Although though numerous fungi, notably *Aspergilli*, are known to generate various families of enzymes, choosing a specific strain is still a laborious process, especially when economically viable enzyme yields need to be attained. In the current study, strains of *A.niger* and *A.flavus* were screened to find the native, productive alpha-amylase producers among the collection of fungi in the First Fungal Culture Bank of Pakistan [7].

For the purpose of identifying Aspergillus species that produce aflatoxin, an agar medium containing commercial coconut extract and having its pH adjusted to 6.9 was created. When

examined under ultraviolet light, aflatoxin-positive isolates on this medium, but not -negative ones, displayed a distinctive blue or blue-green fluorescence in the agar around the colonies. Within 32 hours of being transferred to the medium, powerful aflatoxin producers started to exhibit fluorescence. During extended incubation, the fluorescence became more intense. Even extremely mild aflatoxin synthesis might be detected after three days of incubation. Some well-known aflatoxin promoters either had no impact or reduced fluorescence output [8].

1.2 Categorization

Aspergillus is an anamorphic genus with over 250 recognized species.

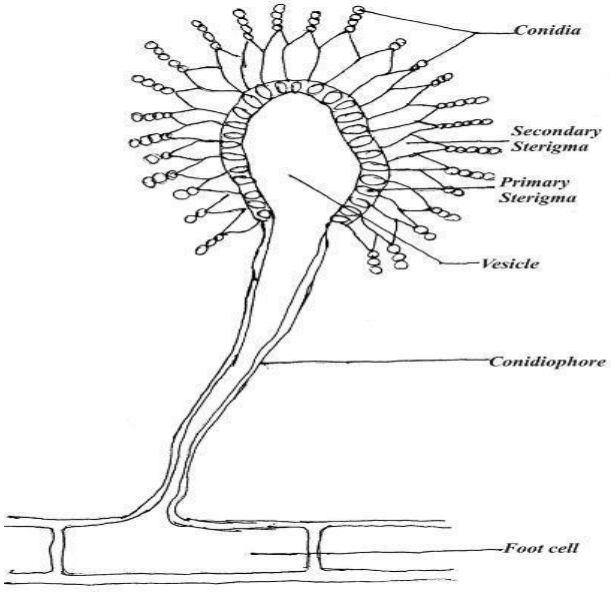


Figure 2- Basic structure of fungus

Adapted from- A. O. Ogunleye and G. A. Olaiya, "Isolation, Identification and Mycotoxin Production of Some Mycoflora of Dried Stockfish (Gadus morhua)," *Academic Journal of Science*, vol. 4, pp. 345–363, 2015.

The genus's teleomorphs, that are usually cleistothecial ascomycetes with ascospores organized erratically in dehiscent asci, are produced by a few of its species. There are various divisions within the genus. In the Flavi section, Aspergillus flavus is classified. This section includes the commercially important aflatoxin-producing fungus *A. flavus* and *A. parasiticus. A. nomius, A. pseudotamarii, A.bombysis,* and *A.parvisclerotigenus* are the less common species that produce aflatoxin in this region. Aflatoxin is produced by four species that are not included in section Flavi: *A.ochraceoroseus, A.rambellii, Emericella venezuelensis,* and *Emericella astellata*..

Anamorphs are asexual phases of Aspergillus that can exist in the later two species, Section Flavi contains several economically important species, including the industrial/food fermentation species *A. oryzae* and *A. sojae*. These two fungi are molecularly related to *A. flavus* and *A. parasiticus*, however they are physically distinct and do not manufacture aflatoxin.

Aspergillus flavus grows for 7 days in the dark at 25 °C on Czapek yeast extract (CYA), CYA with 200 g sucrose, or malt extract agars to produce yellow-green colonies 65-70 mm in

diameter. It works well at 37 °C. A conidiophore's stipe is typically 400-800 m long and has a rough wall. The vesicles have a diameter of 20-45 m and can be globose or elongate in shape. Regardless of seriation, at least 20% of aspergilla on CYA produce both metulae and phialides. Conidia have smooth to coarsely roughened walls and can be globose to ellipsoidal in shape (Fig. 2). Some strains produce sclerotia, which are black-colored, long, firm-walled structures. These are typically 400-700 m in diameter.

Aspergillus flavus looks similar to several other species. *A. oryzae* differs from *A. flavus* in that it produces more floccose colonies that mature to brown on CYA and larger (4-8.5 m) conidia. Colonies of *A. parasiticus* are frequently darker green, with very rough conidial walls. *A. bombycis* grows slowly (0-37 mm) on CYA at 37 °C and has smooth-walled stipes. *A. nomius* differs from *A. flavus* in its mycotoxin profile: *A. flavus* produces only B aflatoxins (see below), but *A. nomius* produces both B and G aflatoxins. *A. parvisclerotigenus* has 400 million sclerotia and produces both B and G aflatoxins often. Differentiation can be accomplished by a variety of molecular approaches.

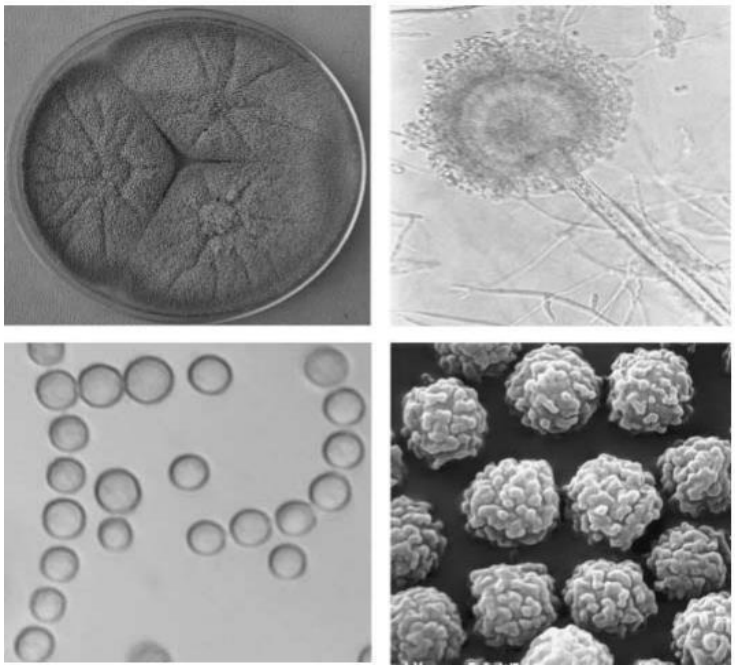


Fig. 3a,3b,3c,3d- Conidiophore of *A. flavus*, scanning electron micrograph of *A. flavus* conidia, conidia as seen under the light microscope at 100×, colony of *A. flavus* after incubation for 7 days at 25 C on CYA agar, and conidia as seen under the light microscope.

Adapted from- M. A. Klich, "Aspergillus flavus: the major producer of aflatoxin," *Mol. Plant Pathol.*, vol. 8,no. 6, pp. 713–722, 2007.

1.3 Aflatoxin detection methods

Before aflatoxin was identified, a relationship among *A. flavus* infection and seed BGY fluorescence under ultraviolet light was well established. Although this technique is use to identify possible aflatoxin contamination, it is not always reliable. While BGY seed may not be contaminated, non-fluorescent seed may. This molecule was not discovered as a kojic acid dehydrogenator dimer linked at the C-6 locations until 1999. The fungus creates the kojic acid component of the BGY, which combines with the peroxidases of the host plant to make the chemical.

Accurate estimation of aflatoxin contamination levels became important after the threat of aflatoxin was identified. Aflatoxin levels were measured and methods for chemical extraction, chromatographic purification, and purification were devised (Pons and Goldblatt, 1969). Due to its simplicity and low cost, thin layer chromatography has now established itself as the gold standard for aflatoxin analysis and screening. Other methods used include gas chromatography, HPLC, and other techniques such the enzyme-linked immunosorbent test (ELISA). Official validation procedures for analysis were issued by the Association of Official Analytical Chemists in 2006. The development of new methods based on PCR, infrared and hyperspectral imaging, is underway (Bhatnagar et al., 2004).

It is not unexpected that the sample stage is the most unpredictable in aflatoxin testing because aflatoxins are not evenly distributed in agricultural products (CAST, 2003; Coker, 1998). While there are various steps that can be done to reduce variation, there is currently no global consensus on sampling procedures for any crop (Whitaker, 2001).

1.4 Factors Affecting the Formation of Aflatoxin in the Field

Row crop field contamination is frequently brought on by drought and temperature stress. Drought stress and temperatures of 29 °C caused the highest number of food grade peanuts to get infested and the highest amounts of aflatoxin, according to experiments with peanuts 85-100 days after planting.

Other environmental confluences led to decreased colonisation rates and no aflatoxin production (Cole et al., 1984). Irrigation can lower the levels of aflatoxin in peanuts, according to a number of investigations (Cole et al., 1989). Cotton with moderately severe drought stress (-1.6 to -1.9 MPa) at anthesis had greater *A. flavus* infection levels in the resultant seeds than cotton with lower or higher water stress conditions during blooming. High heat and drought stress, as in

other vulnerable crops, nearly always precede aflatoxin breakouts in maize.

It is unknown how exactly dryness and high heat contribute to field pollution. Because dryness and high temperatures have an impact on a plant's physiology, plants that are agitated may be more vulnerable to infection or the formation of aflatoxin. Like, plants produce significantly more proline under drought stress, and proline has been found to encourage the synthesis of aflatoxin. The production of numerous phytoalexins, which are antibacterial compounds made by plants, is inhibited by drought stress. Phytoalexin production in drought-stressed plants had to stop before aflatoxin appeared in immature peanuts. Another hypothesis is that in these circumstances, the fungi that typically interact with *A. flavus* in the soil do not develop as efficiently, providing *A. flavus* an advantage. Even among Aspergillus species, *A. flavus* has a greater preferred temperature for growth (25–42 °C) than many others. *A. flavus* is adaptable to a variety of environmental factors. It can grow in water with a potential of -35 MPa, which is lower than the minimum for many other fungal species, even though -2 MPa is the water potential at which it grows best.

1.5 FIELD CONTROL

Either lowering aflatoxin production or inhibiting the fungus can lessen aflatoxin contamination. *A. flavus* doesn't seem to acquire resistance from other fungus. *A. flavus* was not resistant to peanut genotypes that were resistant to other fungi, and they did not reduce aflatoxin. A number of variables affect the fungus's development, spread, and production of aflatoxin. New field control strategies are emerging as a result of the utilization of these characteristics.

a) Cultural practices

Aflatoxin is an issue in stressed plants that can be reduced by reducing plant stress. Many of the farming techniques used to improve quality and output also reduce vulnerability to A. flavus infection and aflatoxin generation. Planting locally suited cultivars, planting at the proper seed density, adequate fertilization (particularly nitrogen), reducing insect damage, controlling weeds, irrigating when necessary, and harvesting as soon as the crop is grown are all examples of these strategies. Unfortunately, stopping the formation of aflatoxin using these practices is not always practicable or adequate.

b) Exclusion from competition

The theory behind this technique is that toxic strains of fungus can be replaced in crop fields by non-toxic ones, filling the void left by toxic strains. Not eradicating mould infestation is

the objective: rather, it is to reduce aflatoxin levels. *A. flavus* is a pathogen that affects both humans and animals, hence competitive exclusion techniques must minimize airborne spore numbers. *A. flavus* produces a number of toxins in addition to aflatoxin, thus caution is also necessary. A considerable decrease in aflatoxin was observed in BGY luminous bolls when non-aflatoxigenic strains of *A. flavus* were applied to cotton early in the growing season. A non-aflatoxigenic strain (AF36) is presently used to control aflatoxin in commercial cotton fields. Aflatoxin levels in peanuts have been successfully decreased using a commercial competitive exclusion product known as afla-guard. In Australia, a similar approach is being investigated for aflatoxin control in peanut-growing regions. Aflatoxin in maize has been reduced using non-aflatoxigenic strains as well, although these have not yet reached the market.

c) Reduction of injury

Aflatoxin was reduced by chemical control of the pink boll worm (*Pectinophora gossypiella*) in cotton. According to Cotton et al. (1997), transgenic cotton containing *Bacillus thuringiensis* (Bt) insecticidal proteins reduces or completely eradicates the aflatoxin problem caused by some insects but not by other entry points. It has been difficult to manage aflatoxin in Bt cotton lines. In peanut, plots treated with pesticide had lower *A. flavus* infection rates. Transgenic peanuts carrying the Bt gene displayed much lower levels of aflatoxin than non-Bt peanuts in an early log-transformed data analysis. Although aflatoxin has not always been resistant to Bt maize, it is frequently linked to insect damage in maize.

d) Host resistance

Early efforts to identify high yielding crop varieties of maize and peanuts resistant to *A. flavus* contamination and aflatoxin formation were mainly unsuccessful. There aren't any commercial cultivars available right now that are totally resistant to aflatoxin contamination. Recently, resistant germplasm in maize has been discovered as a result of proteomics. A number of genes for resistance to *A. flavus* and/or aflatoxin formation or plant stress will be employed to generate resistant lines with acceptable yield. As there are no naturally resistant varieties, cotton plants are being genetically modified to introduce genes that are either antifungal or may reduce aflatoxin synthesis.

e) Molecular control of toxin production

Aflatoxin synthesis has been demonstrated to increase in response to oxidative stress. Aflatoxin production has been found to be inhibited by hydro soluble tannins and gallic acid, both of which are known antioxidants. The molecular elements of this are being studied, and research is being conducted to establish whether using compounds that impair the oxidative stress response can diminish aflatoxin formation.

f) post-harvest options

The best option might be to destroy the crop if a pre-harvest aflatoxin assessment indicates that a field is severely infected. To reduce aflatoxin levels to acceptable levels, another option is to feed the crop to less sensitive animal species or combine infected and uncontaminated crop seed. It should be emphasized that mixing is prohibited in certain nations and permitted in others only under extremely specific conditions. Solvents can be used to eliminate aflatoxins chemically. This approach works well, but it is not economical. Although temperatures employed in heat treatment must be much higher than those used in regular cooking, and effectiveness is reliant on other factors such commodity moisture content, aflatoxins can be reduced (but not entirely removed) by heat treatment.

It's interesting to note that the nixtamalization method, used to make tortillas, involves soaking and boiling corn in an alkaline solution. Radiation has been used to eliminate aflatoxin to varying degrees of success. Adsorbents, ammonization, and sorting are now the three commercially employed techniques. Aflatoxin removal from feeds is possible, for instance, using clays. These goods come in a variety of varieties. This method carries the danger that it might bind nutrients in addition to aflatoxin, however the stated reductions are small. With different degrees of efficacy, a number of additional sequestering substances have also been investigated. Exposing the seed to gaseous ammonia or ammonium hydroxide is the process of ammoniation. About 90% of the aflatoxin is eliminated and transformed into less dangerous molecules. The US FDA has not approved this approach since some of these items are marginally harmful; nonetheless, ammoniation is accepted in other nations and utilized in commodities that are not transported between US states.

Taking out seed that has been contaminated with aflatoxin is another method for reducing post-harvest aflatoxin levels. Seed discoloration in peanuts is usually caused by *A. flavus* and other fungi. In peanut shelling plants, where shelled peanuts are color-sorted to weed out contaminated

seed, this property has been employed. Pitt and Hocking go into great depth about the process used in Australia to get aflatoxin-contaminated seed out of the ground. The method has not shown to be commercially successful for almonds and pistachios.

1.6 The production of aflatoxin: biochemistry and molecular genetics-

It has become possible to clone the genes for the 21 distinct enzymes required to generate aflatoxin. The cluster also contains the *aflR* and *aflJ* genes, which encode proteins involved in the transcriptional activation of the majority of structural genes. All the genes are clustered inside a 70 kb region of the fungal genome, according to restriction mapping of DNA cosmid and lambda phage libraries from *A. flavus* and *A. parasiticus.* [5].

The complete 70 kb DNA sequence or the 25 open reading frames (ORFs) together comprise a firmly established aflatoxin pathway gene cluster. On average, one gene takes approximately 2.8 kb of chromosomal DNA. The fatty acid synthase (FAS) alpha (5.8 kb) and beta (5.1 kb) components are both large genes that are between 5 and 7 kb each (PKS; 6.6 kb), as are the polyketide synthase (PKS) gene. Except for these three large genes, the remaining 22 genes have an average size of about 2 kb. The cluster sequence has a 2-kb DNA region lacking any detectable ORFs at the 5′ end. This sequence clearly indicates that this cluster has reached its conclusion in this route. The 3′ end of this gene cluster is marked by a distinct gene cluster with four genes associated with sugar consumption (33). *A. parasiticus* has been added to the GenBank database with an 82,081-bp fully annotated DNA sequence (nucleotide sequence accession number AY371490), which comprises gene clusters for the aflatoxin pathway and sugar consumption.

Pathway-

Aflatoxins are polyketide-derived secondary metabolites produced via the following conversion path: acetate → polyketide → anthraquinones → xanthones → aflatoxins

Numerous genes participate in the key pathways that lead from early precursors to aflatoxins [6].

Case study

In Kenya, one of the worst outbreaks of aflatoxicosis occurred in April 2004 and resulted in 317 cases and 125 fatalities. The home maize that was infected with aflatoxin that triggered the outbreak was unclear, as was the degree of regional contamination and the state of market maize. The cross-sectional study was conducted to evaluate the level of market maize aflatoxin contamination and the relationship between it and the widespread outbreak of aflatoxicosis. We examined 65 markets, spoke with 243 maize vendors, and gathered 350 samples of maize in the most affected areas. In 55% of the cases, 35% of the cases, and 7% of the cases, there were aflatoxin levels in maize products that were higher than the 20-ppb legal limit for Kenya. Aflatoxin levels in market maize were significantly higher in Makueni than in Thika (geometric mean aflatoxin = 52.91 ppb vs. 7.52 ppb, p = 0.0004), the study district with the highest number of aflatoxicosis case-patients. Maize from local farmers in the affected area was significantly more likely to have aflatoxin levels above 20 ppb when compared to maize bought from other regions of Kenya or other nations (odds ratio = 2.71; 95% confidence range, 1.12-6.59). Widespread aflatoxin contamination of market maize was caused by contaminated household maize that was bought from affected nearby farms and entered the distribution system. Farmers may continue to be exposed to aflatoxin through the purchase of contaminated market maize after their supplies of homegrown maize have run out [8].

1.7 Mechanism of Toxicity:

Before AFB1 can directly interact with DNA, cytochrome p450 enzymes must biotransform it into AFB1-8, 9-epoxide. When this metabolite spontaneously and irreversibly binds to guanine residues, it produces DNA adducts that are highly mutagenic. The activation of the G2/M checkpoint site cell cycle checkpoint breaks down as a result. The A-T mutation causes genomic instability and decreased DNA double strand break repair, which raises the risk of cancer. The G-to-T transversion, which is connected to the high frequency of p53 mutation, is predominantly caused by AFB1. The majority of individuals with hepatocellular carcinoma associated with AFB1 have TP53 mutant DNA, which suggests both AFB1 exposure and hepatic cancer risk [9]. The most common mutation in the p53 gene, codon 249, is associated with human hepatocellular carcinoma (HCC) in communities with significant dietary aflatoxin B1 consumption. Schistosomiasis is known to cause P53 mutation [10]. AFB1, the mostpotent naturally occurring aflatoxin among those known to cause liver cancer, is produced by

A. flavus and *A. parasiticus* and contaminates agricultural items like maize and peanuts both during their growth in the field and during storage following harvest [10, 11]. After consumption, AFB1 is converted by liver cytochrome P450 enzymes into the reactive intermediate AFB1-8,9- epoxide. The quantitatively abundant AFB1-DNA adduct 8,9- dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-Gua) is created when this intermediate conjugates on the N7 atom of deoxyguanosine in DNA. This first DNA adduct is chemically unstable because of the positive charge on the imidazole ring, favouring reconfiguration to the open ring form of AFB1-formamidopyrimidine (AFB1-FAPY) or depurination to create an abasic site (AP) [12].

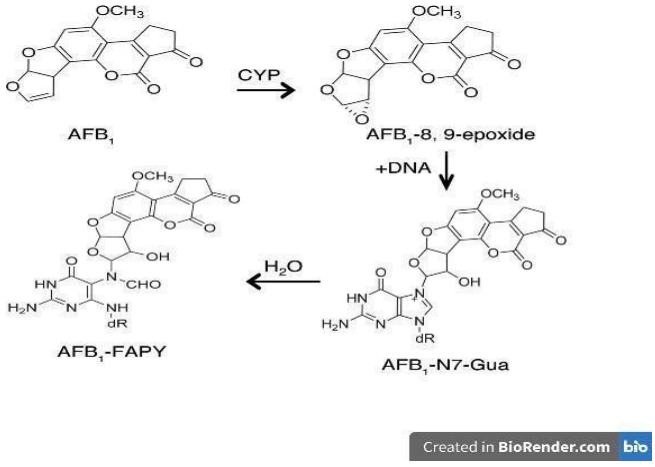


Figure 4- The AFB1-DNA adducts' chemical structures were created using biorender.

AFB1-N7-Gua-induced Mutagenesis-

Mutagenesis, which is defined as the replication of a damaged DNA template in tumour suppressor genes or oncogenes, is thought to be the first step in the development of cancer. More than half of the HCC samples showed a G to T mutation at codon 249 of the p53 tumour suppressor gene (AGG), according to analyses of two surveys carried out in regions with a high prevalence of HBV infection and aflatoxin exposure [13, 14]. Due to its detection in HCCs other than those exclusively linked with HBV, this mutation serves as a diagnostic of aflatoxin exposure. Numerous investigations using experimental mutagenesis have revealed that the G to T transversion mutation is the most common one brought on by exposure to AFB1 [15, 16].

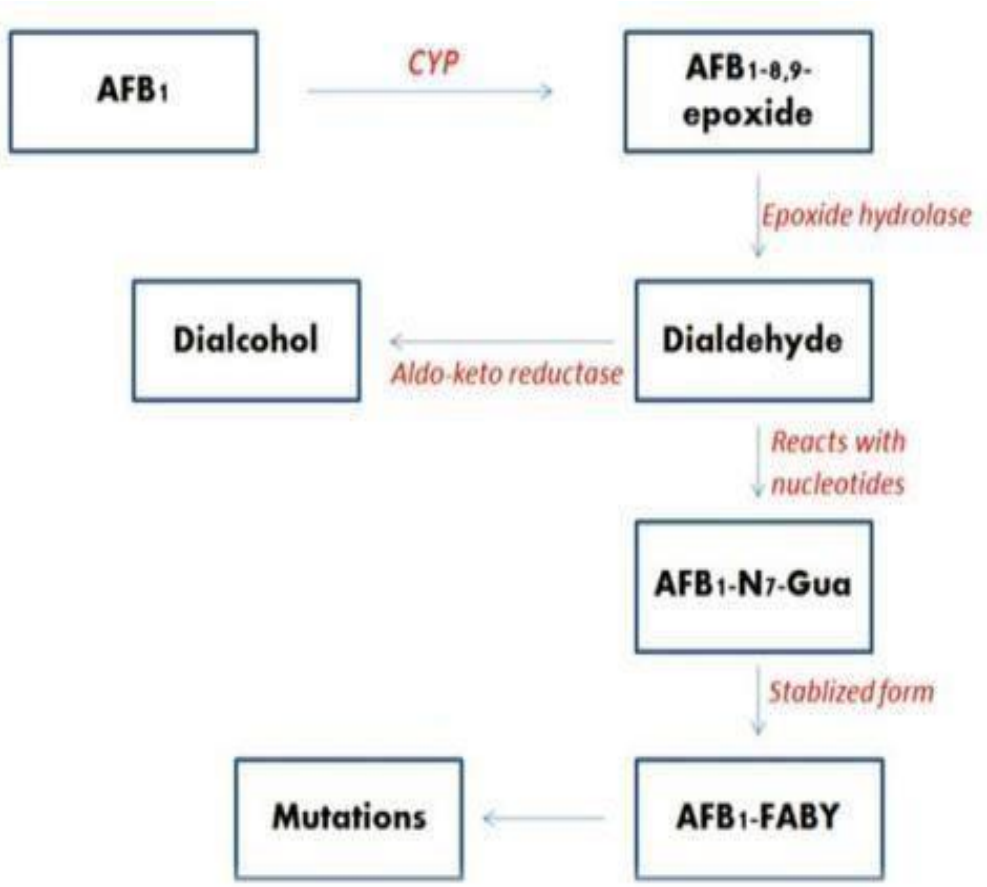


Figure 5 -Mechanism of toxicity of AFB1.

Adapted from- A. Bansal, M. Sharma, A. Pandey, and J. Shankar, "Aflatoxins: Occurrence, Biosynthesis Pathway, Management, and Impact on Health," in *Fungal Resources for Sustainable Economy: Current Status and Future Perspectives*,2023, pp. 565–594.

CHAPTER 2

PREPARATION OF AGAR SLANT, NUTRIENT BROTH AND AGAR PLATE

Media Preparation:

Materials required-

- Test tube
- PDA
- Spatula
- Beaker
- Autoclave
- cotton plugs
- Aluminum foil
- Weighing balance

Determine the total amount of medium required. Specify the number of slants and brothrequired. Using a dry spatula, weigh the amount of medium needed. Because of the medium's hygroscopic nature, avoid excessive exposure to the environment. Fold the foil at once and label with marking pen.

Carefully transfer weighed powder into flasks. Wash off any sticking powder withdistilled water into the flask.

Add required volume of distilled water, swirling the flask slowly

Melt the agar in microwave oven

For making slants, dispense melted agar per test tube using a pipette or a glass syringeand place the cotton plug. For making broth, dispense the melted medium into the tubes.

- Put test tubes together and cover with aluminum foil or place in autoclavable plastic so as to protect cotton plugs from getting wet which may subsequently cause contamination.
- The culture media should be sterilized right away.
- After sterilization, lay sterile test tubes containing agar medium across slanting

boardsand allow them to solidify. Let the sterile broth tubes cool completely.

- Refrigerate the sterile media for future use

Figure 6- Slants were prepared from the culture available in stock for further process.

REVIVAL OF CULTURES

Active cultures

Subculture the organism to the suggested medium right away, and then incubate it under the advised temperature and aerobic/anoxic conditions as detailed in the data sheet. The MTCC inventory of strains includes details on each culture's growth medium and environmental conditions.

Freeze – dried cultures

Since the contents of the ampoule are in a vacuum, care should be taken when openingit.

1) Make a mark on the ampoule close to the centre of the cotton wool with a sharp file.
2) To disinfect the region around the mark, use alcohol.
3) Break the ampoule at the indicated location after wrapping it in thick cotton wool (or gauge).
4) Remove the ampoule's pointed top carefully. A quick opening will discharge tiny dried-organism particles into the laboratory air whereas a snap opening will drag the cotton plug to one end.
5) 0.4 cc of sterile water should be used to prepare a suspension of fungi, which should then be left to stand for 20 minutes before being transferred to a solid medium.
6) Add a few drops of the suspension to the liquid media that has been suggested (and agar-solidified) in a Petri plate.
7) Incubate at the right temperature and in the ways that the culture has been advised.
8) The culture should start to grow if our directions are faithfully followed within a few days. Some fungi need lengthy incubation times, perhaps as long as 15 days.
9) Prior to disposal, the original ampoule's contents should be sterilised in its entirety.

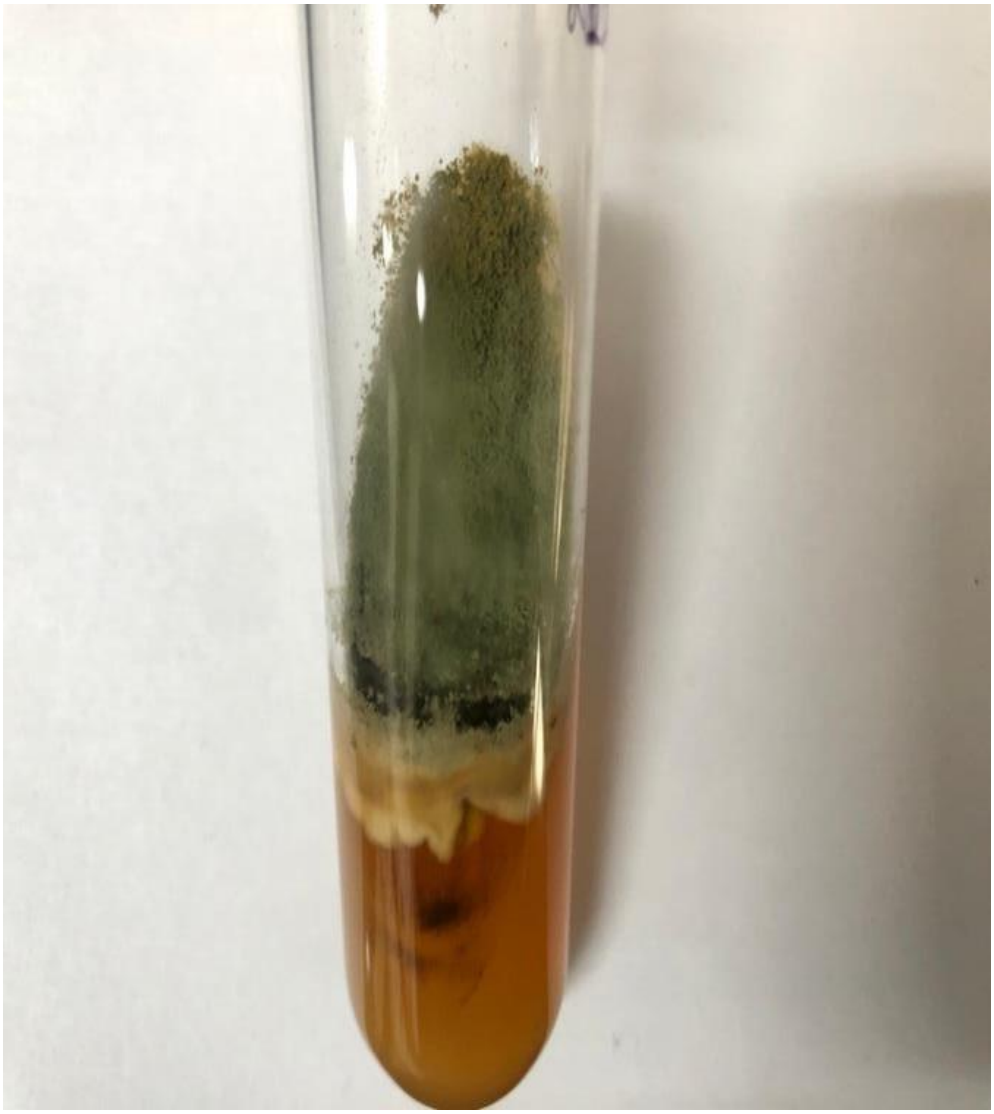
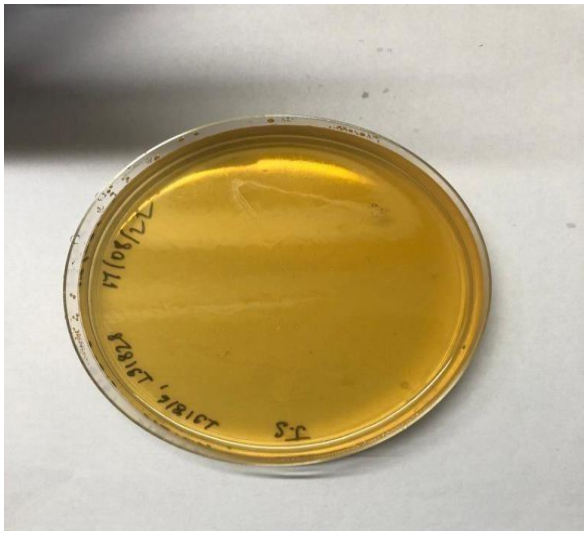


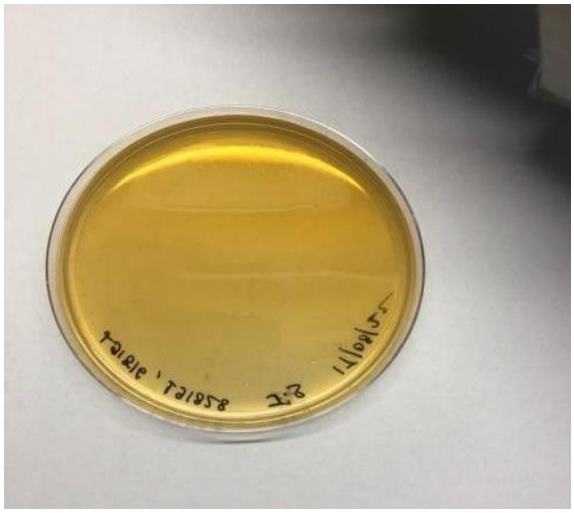
Figure 7- Slant with growth of revived culture after, plating was done under Laminar air flow (LAF)

Strain- soil strain (MTCC. No.9367)

8(a)



8(b)



8(c)

8(d)

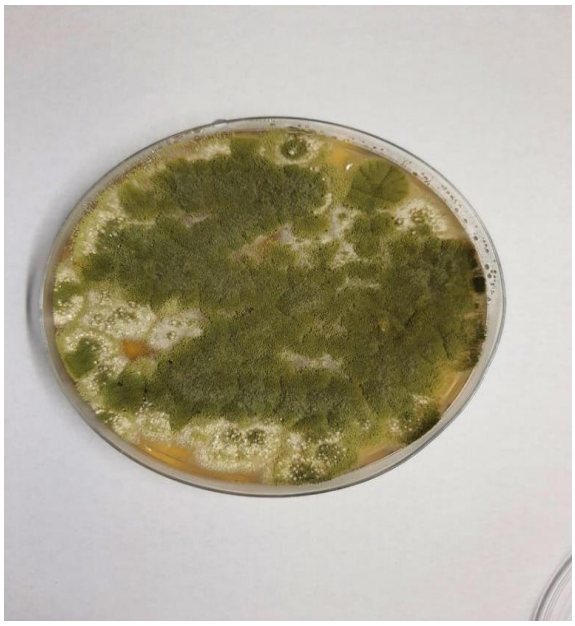
Figure 8a- work done under Laminar Air Flow.

8b- showing procedure of plating, streaking strain BT-03

8c- showing procedure of plating, streaking strain BT-05

8d- growth of fungi on the plate after 24 hrs.

9(a)



9(b)

9(c)

9(d)

Figure 9a,9b,9c,9d- shows streaking of *A. Flavus* strains which was contaminated with some unknown strain of other or same fungi.

1) Culture condition:

Coconut, which is typically used on a daily basis. This specific food product was chosen as the host since it is often preserved for an extended period of time. Coconut that is maintained for an extended period of time loses both quality and quantity. *A. flavus* is one of the most prevalent mycotoxin-producing pathogens, and it is regularly impacted by both abiotic and biotic factors. As a result, it is utilized as a host in this study to examine the fungal growth signaling system.

2) Isolation of pure isolates:

Conidia harvesting

Conidia are asexual propagules that develop in many bacteria. Sporangia, which are sac- like structures, generated asexual propagules. Conidia are exposed to the outside world after maturity in sporangia. Conidia have the ability to germinate and produce a new generation.

Conidia must be gathered because they produce a clean colony of matching conidia spores that are easy to handle and may be used to inoculate appropriate media. Conidia are best extracted with TWEEN from a phosphate buffered saline solution [17].

Chemical Reagents:

A) Phosphate buffer saline Table 1:

Composition of PBS

S.No.	Chemical name	For 1000ml
1	Sodium chloride (NaCl)	8.0 g
2	Potassium Chloride (KCl)	0.2 g
3	Sodium phosphate, dibasic anhydrous ($Na_2HPO_4.2H_2O$)	1.44 g
4	Potassium phosphate monobasic (KH_2PO_4)	0.24 g
5	Distilled water	1000 ml

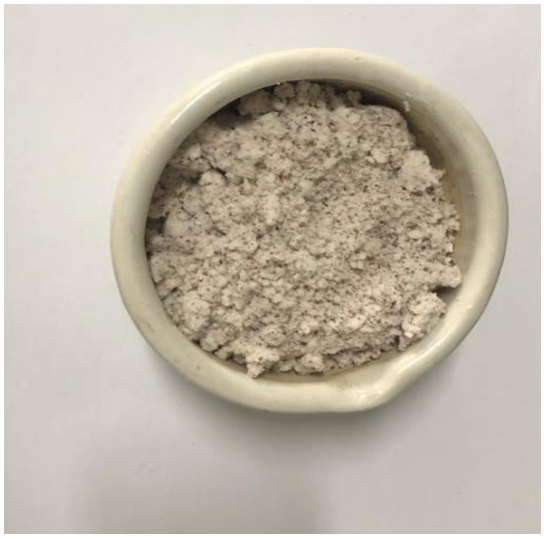
Phosphate buffer saline tween

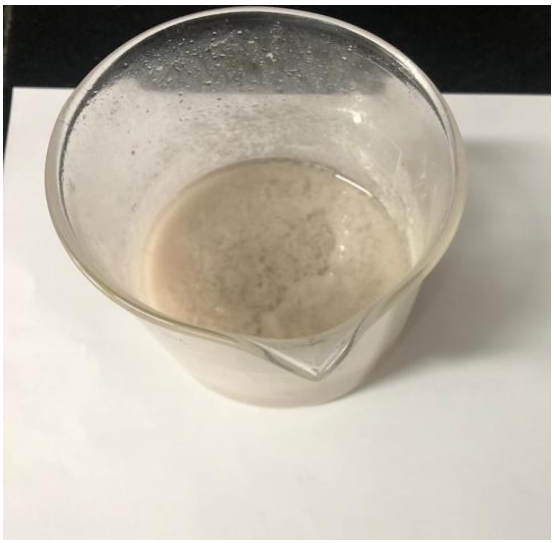
The PBS was created by combining 100 ml of PBS with 200 l of tween detergent and autoclaving the mixture.

Note: Keep headroom to avoid overflowing during autoclave and do not completely mix it to avoid foam development.

Procedure:

- A plate with fully formed conidia was taken.
- The culture received 5-6 ml of PBS.
- The PBS was carefully combined to extract only its top conidia portion without damaging.
- In Eppendorf, approximately 2ml of the mixture was collected.
- The Eppendorf was then centrifuged for 5 minutes at 4°C at 10,000 rpm.
- After discarding the supernatant, the pellets were collected.
- Each Eppendorf tube received an identical amount of PBS, which was added and properly mixed.
- At 4°C, it was centrifuged once again for 5 minutes at 10,000 rpm.
- The supernatant was removed once more, and an equivalent amount of PBS was added.
- This was then kept for a later use.





10(a) 10(b)

Figure 10(a)- preparation of coconut media using dry coconut and separate the fiber portion

10(b)- preparation of coconut media using dry coconut and separate the filtrate portion used it as Media.

3) Subculturing:

Microorganisms must be subculture in an environment that mimics the microbe's true growth conditions in vitro. The medium composition was changed and modified to allow cell development in- vitro. Subculturing is accomplished by placing a single colony of cells in a new plate. Subculturing is necessary because the medium's toxic metabolites build up over time, there is a nutritional shortage, and more cells are produced as a result of growth, all of which eventually stop the medium from supporting additional cell development. Future cell growth is eventually constrained by the rise in cell number brought on by growth. For the correct passage number to be recorded, subculturing isalso required. In Potato dextrose agar (PDA) plates, *Aspergillus flavus* was sub cultured. Fungus culture was carried out on PDA plates. It is a generic media for growing fungi that includes additionssuch as antibiotics to assist suppress bacterial development. It is made up of potato infusion (dehydrated) and dextrose, both of which promote fungal development.Agar is the solidifying agent. To prevent bacterial growth, chloramphenicol can beadded. Potato dextrose agar: pH should be maintained at 5.6±0.2

Table 2: Composition of PDA

S.NO	CHEMICAL NAME	For 1000ml
1	Potato infusion/potato extract	200g/4g
2	Dextrose	20g
3	Agar	20g
4	Distilled water	1000ml

Procedure:

- Conidia were extracted from previously obtained vials.
- 200L of conidia were collected and seeded into new PDA plates.
- Plates were maintained at 37ºC in an incubator.

Microscopy-

Lacto phenol cotton blue (LCB)

Staining

- A clear glass slide was taken
- An LCB drop was installed on the slide.
- A fungal growth was removed off the PDA plate and placed on a drop of LCB.
- The fungal culture was then gently teased with LCB.
- The cover slip was put and left undisturbed.
- After that, the prepared slide was examined under a microscope at magnifications of100, 400 and 1000.

Preparation for infecting Maize-

Serial dilution:

- 100L of previously held conidia in PBS were transferred to the new vial, along with900L of PBS.
- This serial dilution procedure was repeated 10-6 times.

Spore counting:

To count the quantity of cells or spores in a sample solution, a hemocytometer is utilized. It is a tool that makes counting the number of spores in a solution easier. Under a microscope, the cells per chamber could be counted thanks to measurements of the space between the lines and the depth of the chamber.

Formula for hemocytometer cell counting:

a) Determine the number of spores in each sizable chamber in the corner.
b) It is calculated how many spores there are overall.
   Spores/ml = Average spores counted $*10^4$.

Procedure:

- 70% ethanol was used to clean the hemocytometer.
- The hemocytometer's cover slip was appropriately positioned.
- Cell suspension was administered from the hemocytometer's edge.
- A hemocytometer was studied using a microscope.
- The number of spores in each square was counted.

Infection of maize:

- The total number of seeds collected for infection was tallied.
- Autoclaved distilled water was used to soak the seeds for 3 hour
- The seeds were autoclaved after soaking.
- In two different plates, 1ml of suspended conidia from 106 dilutions were dispersed over autoclaved seeds.
- Plates were maintained at 37ºC in an incubator. Fungus development on the seeds was noticed on the third and fifth days

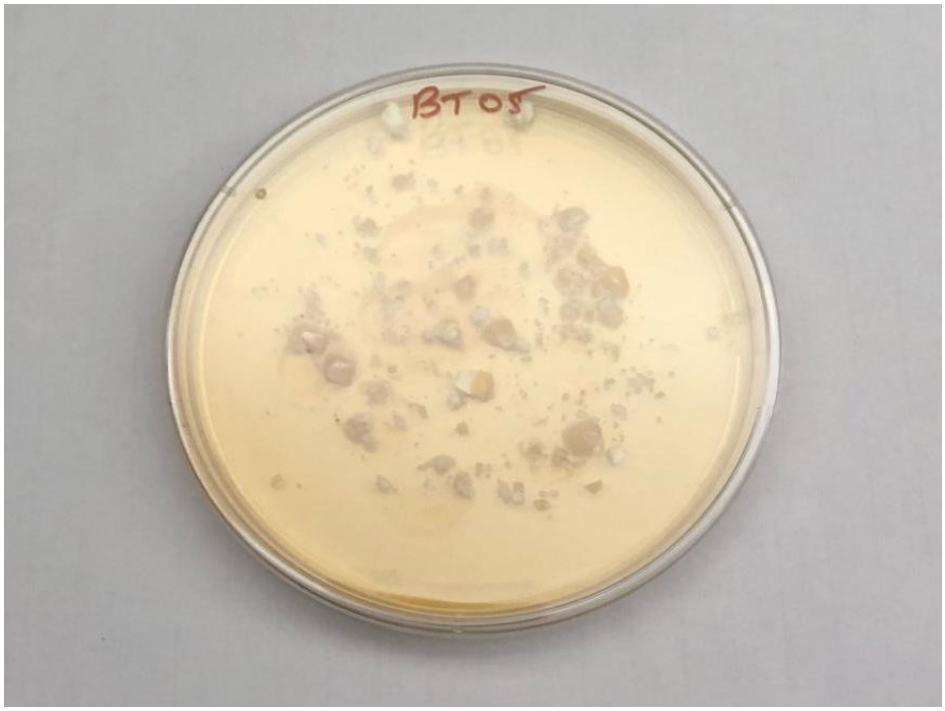


Figure 11(a)- Maize (Sterile) infected with *A. flavus* strain BT05 using PDA media at 0 hour

Figure 11(b)- Maize (Sterile) infected with *A. flavus* strain BT03 using PDA media at 0 hour

Figure 12(a)- Maize (Sterile) infected with *A. flavus* strain BT03(MTCC- 11588) using PDA media before 24 hours

Figure 12(b)- Maize (Sterile) infected with *A. flavus* strain BT05 (MT-11866) using PDA media before 24 hours

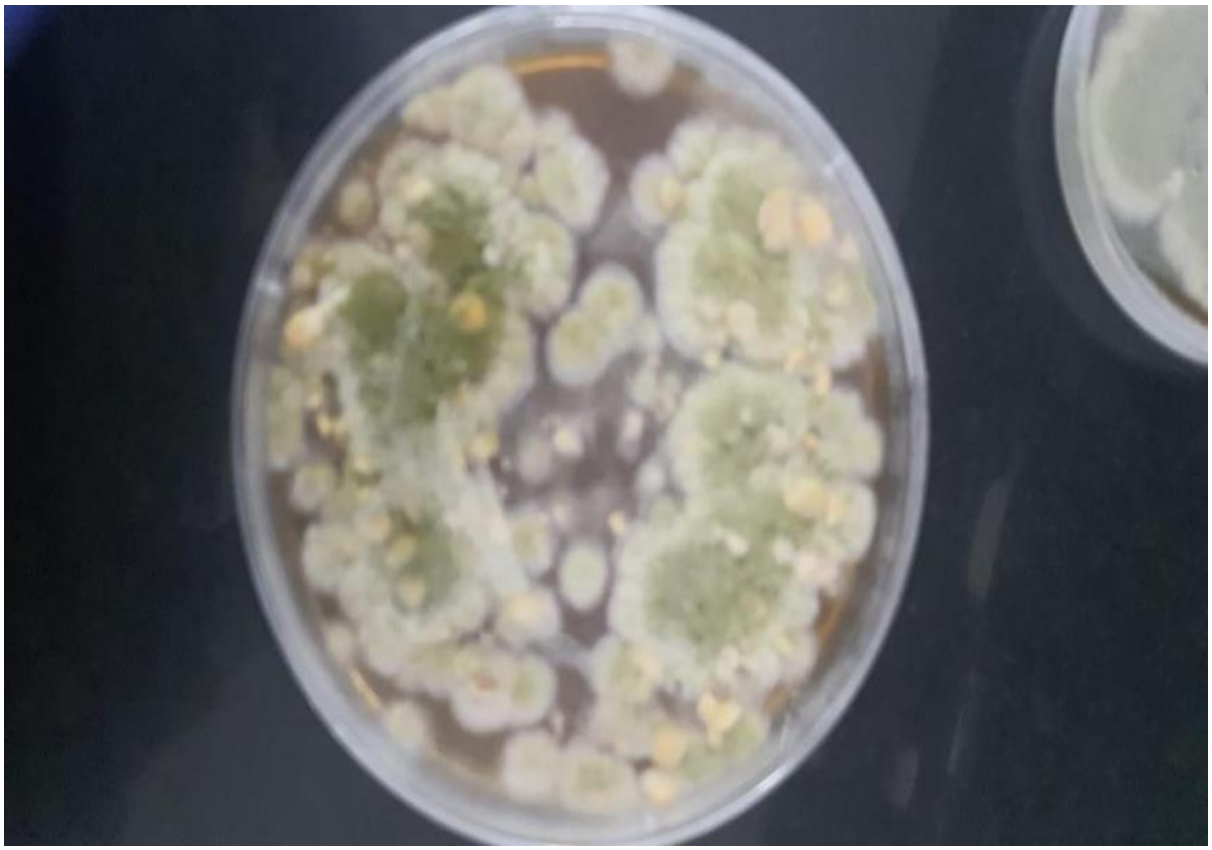


Figure 13- Infected maize Co-culture observed after 72 hrs.

Same procedure first done using PDA media and now trying same set up with Coconut media.

DNA Extraction Methodology:

Steps-

1)	20mg mycelia powder
2)	600µl extraction buffer
3)	600µl phenol to chloroform (1:1)
4)	Centrifuge 12500g for 5 mins
5)	Collect the upper layer (aqueous phase ‘x’ µl)
6)	‘x’ µl chloroform: isoamyl
7)	Centrifuge 12500g for 5 mins
8)	Collect the supernatant
9)	Add 0.7 V of 2 propanol
10)	let the pallet dry
11)	40 µl sterile H2O
12)	Add RNAase
13)	Incubate at 37º C for 3 hrs.
14)	Store at - 20ºC

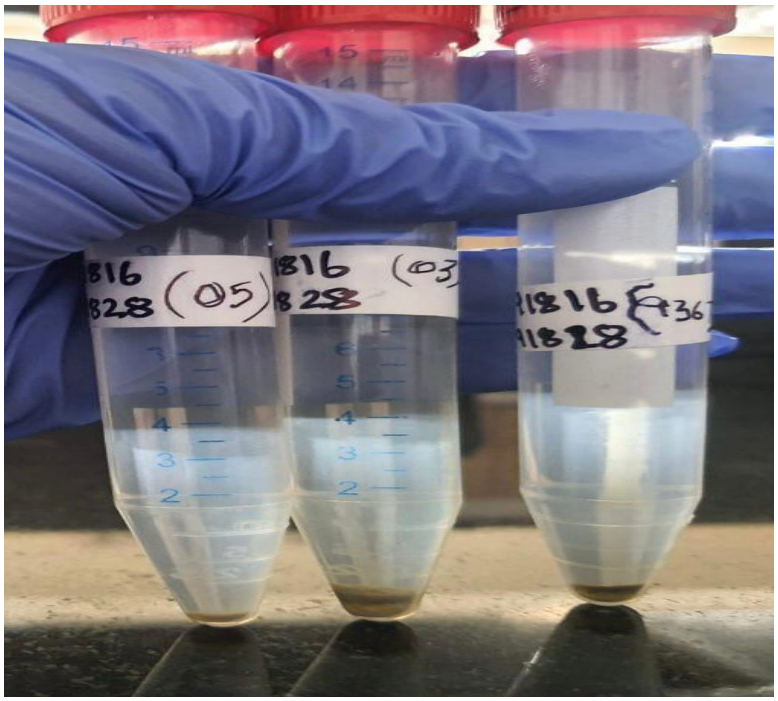


Figure 14-Spores collected for DNA extraction

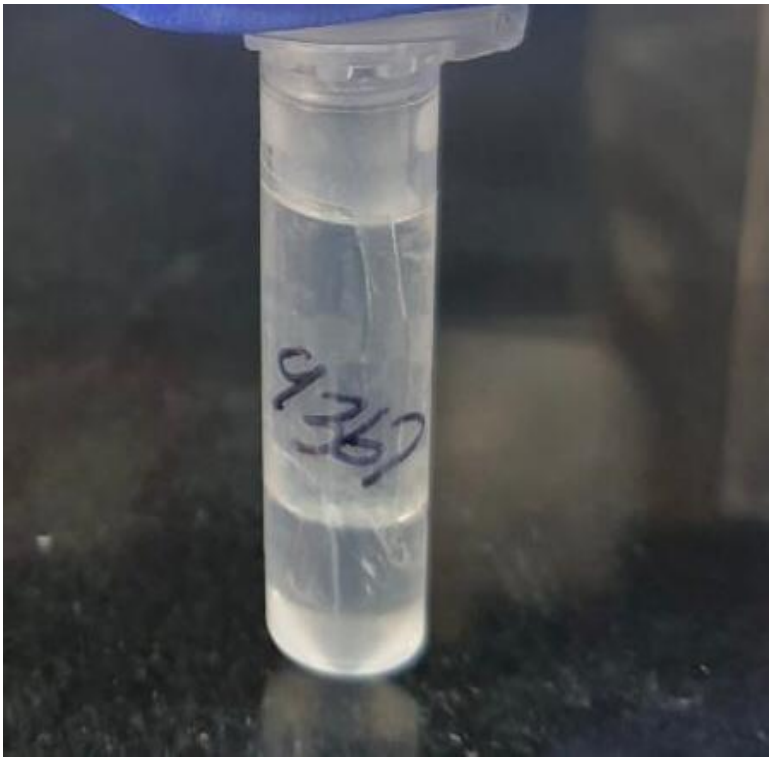


Figure 15- during DNA extraction process differentiation of the phases.

CHAPTER 3

3.1 Method for Screening Aflatoxin-Producing Molds by UV Transilluminator

UV light was used to assess aflatoxin absorption in agar media. Mycotoxin fungi appeared as grey or black colonies in the UV visuals, whereas aflatoxin-producing moulds were white colonies. Cellophane transplantation studies and silica gel thin-layer chromatography revealed that the chemicals that absorbed UV light considerably were largely aflatoxins B1 and Gl released into the agar media by the mould mycelium. UV absorption was not observed when aflatoxin-inducible carbon sources were used instead of glucose in the agar media. Aflatoxin inhibitors such as dichlorvos and dimethyl sulfoxide also dropped UV absorption intensity. These findings imply that this technology could be used to screen for aflatoxin-producing moulds in a simple, safe, and time-efficient manner.

UV photo can be used to identify aflatoxins-producing mould because the absorbance spectra of aflatoxins produced by moulds have peaks at about 362-nm wavelength [18]. This method is a simple technique with no drawn-out extraction or purification procedures, and it is extremely safe because the test moulds are kept on an agar plate during the research, and results can be obtained after a brief incubation period. As a result, this screening method is more practical than the other screening techniques indicated above for research of the biochemical or genetic routes of aflatoxin synthesis that demand a large number of tests. In this study, we also look at how altered experimental conditions affect results.

Culture technique:

- Solid agar media was seeded with a microliter of spore suspension (400 conidia).
- Precisely test the changes in UV absorption induced by aflatoxin formation, the
- aflatoxin-producing strain was inoculated alongside the aflatoxin-producing strain as a control.
- Test solely the changes in UV absorption induced by aflatoxin formation, the aflatoxin producing strain was infected alongside the aflatoxin-producing strain as a control.

Results –

Photography includes fluorescence imaging, ultraviolet photography, and regular natural light photography. On black velvet, petri dishes were placed upside down. Plates were then placed on a UV transilluminator and examined with UV light.

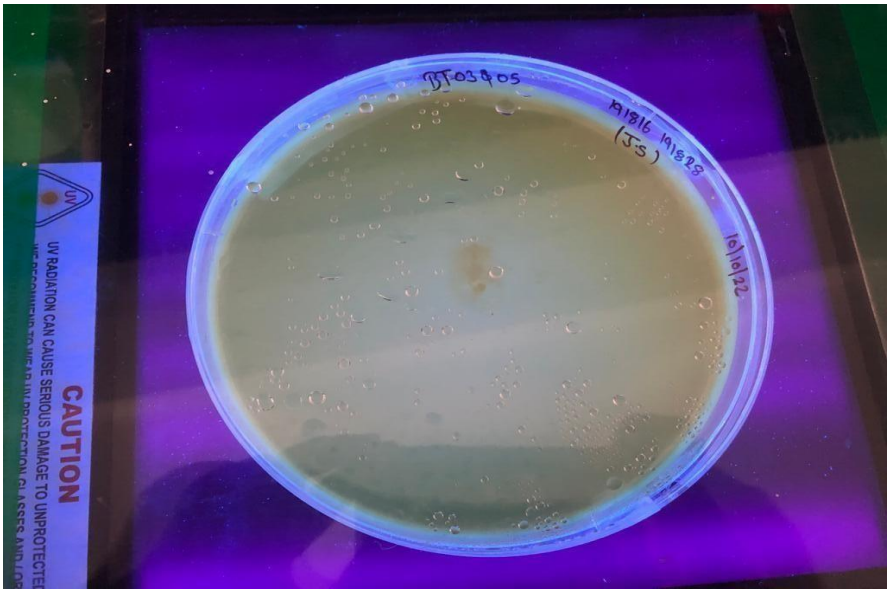


Figure 16(a)- Observe the strain BT03 under UV transilluminator before 24 hrs

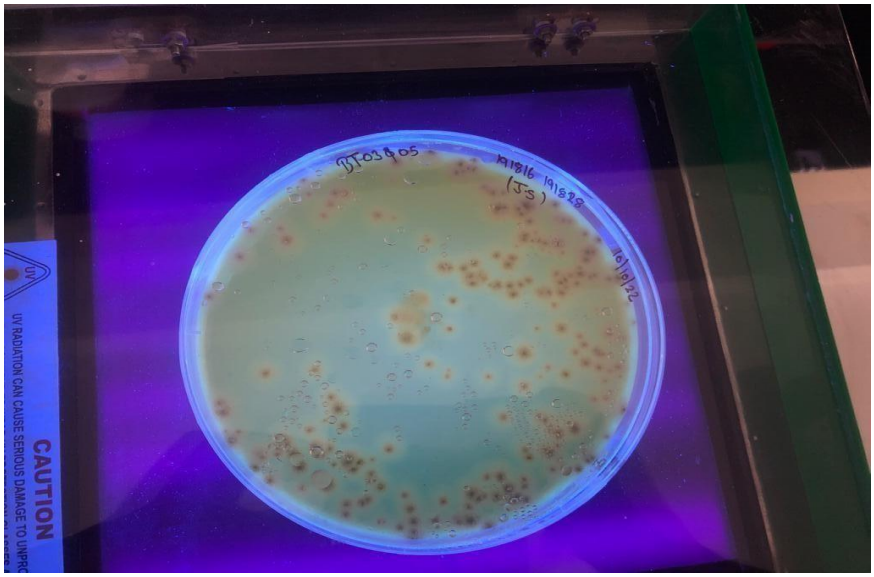


Figure 16(b)- Observe the strain BT05 under UV transilluminator before 24 hrs

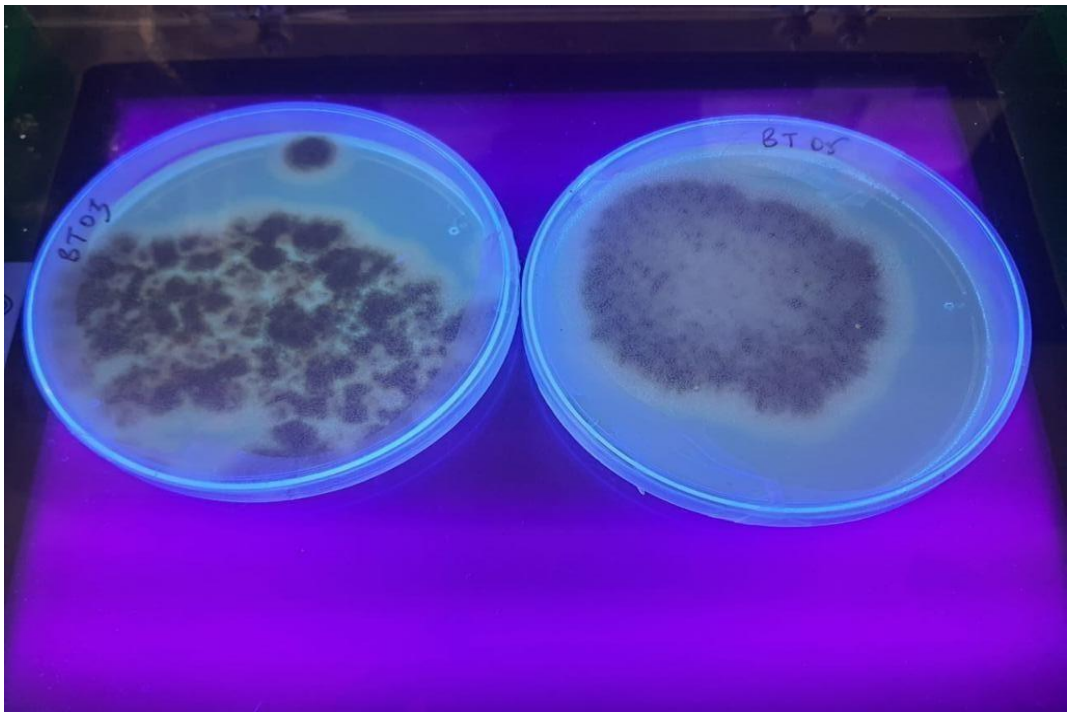


Figure 17- both the strains BT05 & BT03 observed after 48 hrs

Byusing PDA, we are unable to see desired results, so we use different media.

Using coconut media-

For the Baker's and Tropical brands of coconut purchased locally, 100 g of finely chopped coconut was blended for 5 minutes with 200 mL of distilled lukewarm water. For the coconut, 300 ml of hot distilled water was combined with 100 g of finely chopped coconut and homogenized for 5 minutes. The homogenate was always filtered through four layers of muslin, and the pH of the clear filtrate was then raised to 7 using 2N NaOH. The solution was heated to boiling before being chilled to around 50°C, then agar (20 g/liter) was added. After testing the pH, it was brought down to 7 if necessary. Then, while being stirred, the liquid was put onto sterile petri dishes after being autoclaved for 18 minutes at 15 lb/in2 and chilled to 40 to 45°C [19].

CHAPTER - 4

4.1 BIOCONTROL

To protect food and livestock feed from aflatoxin contamination by A. flavus, alternative management strategies for peanuts are applied, such as treating peanuts with fungicides, fumigants, or biocontrol agents. Although employing fungicides to manage A. flavus is a successful strategy, it has a number of drawbacks, such as the emergence of fungicide-resistant strains of the disease and fungicide residues that damage the quality of food and cause environmental contamination [20]. The creation of effective biological controls that pose less of a threat to people and the environment is therefore a top goal. Due to their biological activity and diversity, particularly their ability to synthesise novel chemical compounds with enormous commercial potential, microorganisms have garnered a lot of attention in recent years. Gram-positive bacteria called Streptomyces spp. have been linked to the manufacture of over 7,500 bioactive compounds, including antibiotics, vitamins, and anticancer medications. They can be found in soil, on plants, in air dust. As phytopathogen biocontrol agents, numerous Streptomyces species have been studied with good results. Aspergillus growth and development are strongly inhibited by a number of drugs and chemicals, including certain antibiotics and chitinases produced by Streptomyces species. Additionally, Streptomyces-produced antibiotics like Blasticidin A and Dioctatin A can stop Aspergillus Shakeel, 2018 95% from producing aflatoxin. B. subtilis UTB1, which was previously discovered in Iranian pistachio fruits, demonstrated antagonistic action against A. flavus. In nutritional broth culture and pistachio nuts, this strain has been shown to reduce aflatoxin B1 levels by 86% and 95%, respectively. Despite being able to break down aflatoxin, strain UTB1 has minimal ability to stop fungus growth. The study aimed to enhance the antagonistic activity of strain UTB1 against A. flavus R5. Manufacturers of biocontrol agents and/or antifungal metabolites employ chemical or physical mutagens (such as gamma or UV radiation) to cause mutations in order to increase the effectiveness of their products. Random mutagenesis has been found to increase the antagonistic potential of biocontrol agents (*Trichoderma spp*. and *Gliocladium spp.)* [21]. Employed random mutagenesis (low-energy ion beam implantation) to create mutants of B. subtilis strains that were more effective than the original strain against Rhizoctonia solani and Gibberella zeae, respectively, in the manufacture of antifungal lipopeptides. They used gamma irradiation to perform random mutagenesis on B. subtilis UTB1 to make it an effective A. flavus controller. The goal of the study was to identify a *B.* subtilis strain with enhanced biocontrol capabilities, strong in vitro and in vivo antifungal activity against A. flavus, and high aflatoxin degradation in pistachio nuts.

4.2 EFFICACY OF BIOCONTROL BY NON-AFLATOXIGENIC ISOLATES OF *A. FLAVUS*

More than 100 nations have implemented or proposed laws governing aflatoxin levels in feeds and foods [22]. Because these levels are so low, the regulations impose a significant burden on grain destined for export. Table 1 lists some of these needs. Normally, maize has low aflatoxin levels and meets these requirements [23], but contamination levels can exceed 100-200 ppb in years with substantial A. flavus outbreaks [24]. Aflatoxin levels in cottonseed grown in Arizona frequently exceed the allowable levels for commerce, necessitating remediation either diluting the contaminated meal with less contaminated grain or through chemical treatment to remove aflatoxins. Aflatoxins concentrations after treatments with various biocontrol A. flavus and crops are shown in the table, which summarises results from multiple laboratories. Some investigations found that treated fields had a 20-fold lower aflatoxin level than untreated fields [25]. These are results from experimental laboratory research in which treatments were most likely carried out under optimally controlled and highly controlled conditions. In general, a 5- to 20-fold reduction in aflatoxin levels would be sufficient to allow the crop to meet consumption requirements; but, if the starting levels are extremely high, even a 20-fold reduction may not be enough. Human aflatoxin poisoning has lately been recorded in several Kenyan maize-growing districts [26]. Because of improper storage, maize became contaminated after harvest in numerous cases. It is uncertain whether a pre-harvest biocontrol method might prevent such exposure, and there may be simpler and less expensive strategies to prevent post-harvest contaminated maize consumption.

Table 1 | Allowable levels of aflatoxins in foods and feeds.

Country	Limit in PPB
France	0.1–10
Netherlands	0.02–5
Germany	5
Japan	10
Austria	0.2–1
United Kingdom	10
India	30
Malaysia	35
Mexico	20
United States	20

Table 2 | Efficacy of biocontrol treatments.

Crop	Non-AF agent	Range of AF reduction % (treated control)	Reference
Maize	K49	83–98	Abbas et al. (2012)
	Afla-guard	9–75	Dorner (2009)
	Afla-guard	85–88	Dorner (2010)
Peanut	Afla-guard	89–96	Dorner et al. (2003)
	AFCHG2	75	Zanon et al. (2013)
Cotton	AF36	20–88	Cotty and Bhatnagar (1994)

4.3 A SECONDARY METABOLITE ELSE CLUSTERS OF GENE IN *A.FLAVUS*

Aspergillus flavus can also produce dangerous secondary metabolites in addition to aflatoxins. This demonstrates the need for caution when selecting isolates to use as non-aflatoxigenic biocontrol agents (Rank et al., 2012). CPA and Pseurotin, ergot-like alkaloids, as well as indole-diterpenes, aflatrem, paxillenes, paspalicines, and aflavinines, are included in this group of secondary metabolites (Figure 18).

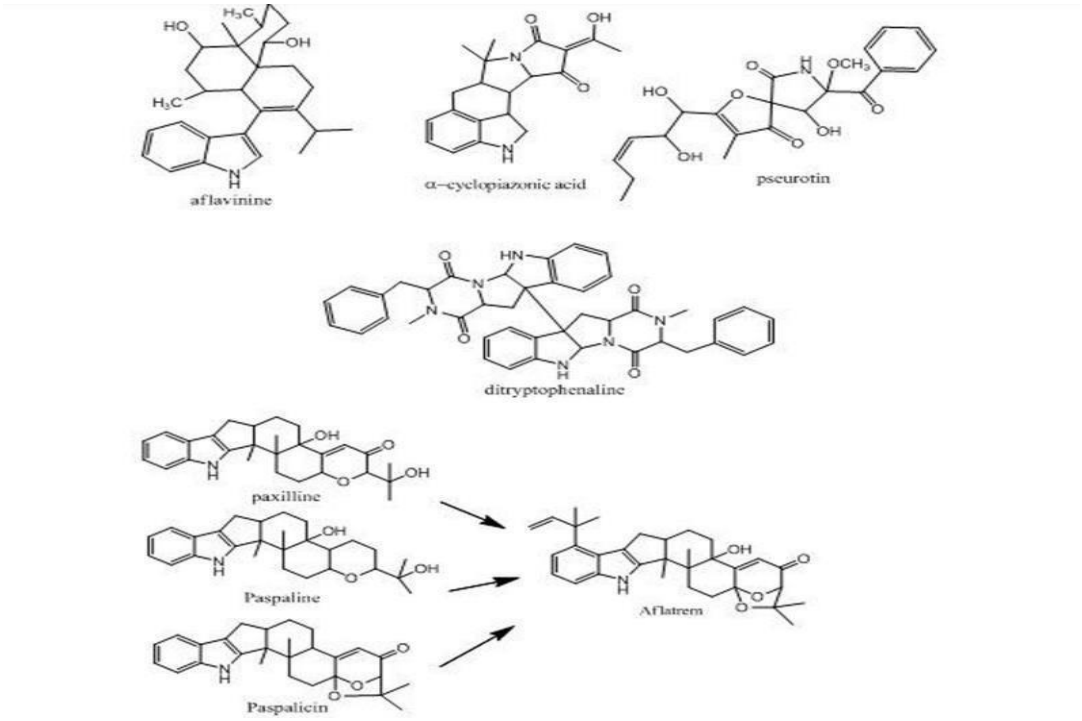


Figure 18-Several *A. flavus* metabolites known to be non-aflatoxic

Animal toxicity studies demonstrate that. these metabolites may possess neurotoxic and nephrotoxic properties, despite the fact that none of them are currently regulated as contaminants in food or feed. Ditryptophenaline, an antagonist of the neurotransmitter Substance P, is one of the frequent metabolites of A. flavus. Recent research has shown that several of these substances developed in greater amounts by the S strain of A. flavus than by the L strain, as well as by some of the competing strains that do not produce aflatoxin. Immune suppression, growth retardation, and liver damage were brought on by eating A. flavus-contaminated maize; the latter of these effects was particularly pronounced in hepatitis C patients [27]. Consumption of aflatoxin has often been held responsible for these negative effects. We suspect that the toxicities documented in those who ate aflatoxin-contaminated maize may have been caused by concurrent ingestion of other hazardous A. flavus chemicals. Recent human poisoning outbreaks in Kenya and Nigeria have been related to the Aspergillus strain S morphotype A.flavus, which may be far more dangerous than the L strain. Additionally, A.flavus is capable of producing metabolites that are not typically thought to be hazardous but could affect animal health. Orcellinic acid, aspergillic acid, and kojic acid are examples of these metabolites, as are iron-chelating siderophores such ferricrocin [28]. What impact these novel metabolites may have on plant and animal health is unknown.

4.4 GLOBAL WARMING AND BIOCONTROL

The Midwest and northern corn-growing regions of the United States and Canada have seen an increase in daily high temperatures as a result of global warming. Soon, temperatures will be similar to those in the southern United States, where maize aflatoxin contamination is a frequent issue. There is currently no known problem with Midwest maize being contaminated with aflatoxin. In addition to temperature changes, global warming can result in climate changes, which can make the weather in agricultural regions more unpredictable. Instances of aflatoxin contamination are more frequent during hot, dry spells, which can harm the host plant and make it vulnerable to infection by A. flavus [28].

As was already mentioned, fungal stress has been associated with increased expression of genes involved in sexual recombination and secondary metabolism production. Aflatoxin contamination outbreaks are frequent in agricultural areas affected by drought, and unanticipated climate changes that cause drought may happen more frequently. Currently, only tropical and subtropical regions of the world (between latitudes 40N and 40S) are affected by aflatoxin contamination of crops [26]. It is possible that by the end of the twenty-first century, the favorable climate for aflatoxin contamination will have expanded to include more of the United States' maize-growing regions, and outbreaks will become more frequent. Since the average global surface temperature has increased since 1901 by 0.8

degrees Celsius, with the majority of that rise occurring in the last 30 years, there is a chance that this increase will have occurred in the last 30 years [27]. Another effect of climate change is that the biocontrol strain can unintentionally injure plants more severely, especially if growing conditions become less favourable for production. Temperature rise-related changes in the soil environment and bacteria could further expose the crop to more harm [29]. Establishing a successful biocontrol strategy requires an understanding of the genetic variation of A. flavus strains; a "one size fits all" approach is unlikely to be successful.

CHAPTER-5

CONCLUSION

- One method for lowering aflatoxin contamination in crops is the use of non-aflatoxigenic *A. flavus* as a biocontrol agent. For this technique to be effective over the long term, though, a number of obstacles must be overcome.
- One of the challenges is the yearly re-application of biocontrol strains, and it is still unclear what will happen to them after one growing season. Research should therefore concentrate on developing methods to guarantee that biocontrol strains remain in the environment and continue to prevent aflatoxin contamination.
- The diversity of *A. flavus* populations presents another challenge and makes biocontrol methods more complex. The production of aflatoxins and other harmful secondary metabolites by *A. flavus* populations varies, and some of these secondary metabolites may be crucial in determining the overall risk associated with consuming contaminated grains. A biocontrol strategy should therefore take into account the variety of *A. flavus* populations as well as any potential impacts of other secondary metabolites.
- Change of climate could potentially complicate the biocontrol effort. Climate change-induced environmental stress may enhance plant sensitivity to the fungus and impair the fungus's capacity to outcross with native *A. flavus* populations. To ensure long-term success, the biocontrol technique should be tailored to changing environmental conditions.
- Lastly, care must be taken to prevent crop loss or harm to the soil microbiota due to the application of *A. flavus* biocontrol. The goal of study needs to be- To develop strategies that minimize potential harm to crops and soil microflora while enhancing the effectiveness of the biocontrol tactic.
- In conclusion, a potential method for preventing the contamination of crops with aflatoxin is the use of non-aflatoxigenic *A. flavus* as a biocontrol agent. To ensure its long-term effectiveness, various issues must be addressed, including the durability of biocontrol strains, the variability in *A. flavus* populations, the impacts of other secondary metabolites, the impact of climate change, and the possibility for crop and soil harm.

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