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Comparative Quantification by HPLC-FLD of Aflatoxin production by *Aspergillus flavus* MTCC 2798

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Aflatoxin is a toxic secondary metabolite generated by many *Aspergillus* species in food crops when kept in hot and humid environments.Aflatoxins are found to be carcinogenic, mutagenic, teratogenic and immunosuppressive in nature and occur more frequently in tropical and subtropical climates, which is same as of India. The contamination of stored food grain with *A. flavus* has been reported in higher amount as compared to other fungal species in India. This type of contamination leads to direct reach to staple food for human being and associated with the disease related to aflatoxin contamination. Since the tolerance limit of aflatoxin is high in human, and hence it is not primarily detected and leads to long term disorder like liver cancer associated with it. The present study focuses on the production of aflatoxin on known MTCC toxicogenic strain of MTCC 2798 *A. flavus* in different semisynthetic and natural media. The aflatoxin B1 is primarily detected by thin layer chromatography and quantified using the HPLC-FLD and the concentration of aflatoxin by HPLC-FLD is found to be higher in rice medium with 35% moisture content as compared to coffee medium with 35% moisture content and SKMY medium. Hence the rice medium can be used as a suitable natural substrate for production of aflatoxin in higher amount as compared to other available synthetic media. Also it is found to be cost effective as compared to synthetic or semisynthetic medium.

Keywords: *A. flavus,* aflatoxin B1, HPLC-FLD, rice medium, SKMY medium

INTRODUCTION

Mycotoxins are fungal metabolites with a relatively high molecular weight that include one or more oxygenated alicyclic rings (Primer *et al.*2001). Mycotoxigenic fungi are prevalent diseases found in all agricultural regions across the world. Mycotoxigenic fungi may grow on a variety of substrates; however the actual reason why fungus prevails on a particular food item is unknown. However, because the nutrients required for their growth, primarily carbon and nitrogen, are abundant in foods, particularly those high in carbohydrates (Kokkonen *et al.*2005).

They may infect and develop on a broad variety of crops, and they are quite versatile in that they can create mycotoxins under a variety of situations, including environmental ones (Richard *et al.*2003).

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Because mycotoxin production does not appear to have a significant biochemical effect on fungal growth, they may have evolved to serve as a defense against a variety of intruders such as insects, microorganisms, nematodes, animals, and humans.Their production can also help to keep the cell's oxidative status at an acceptable level for fungal survival (Reverberi *et al.*2010).For development, germination, and mycotoxin generation, each fungus has an ideal temperature and water activity range. As a result, no one temperature and water activity range can be described as stimulating fungal activity. And, due to differences in environmental conditions and growth requirements, there are obvious differences in fungal development and mycotoxin production across geographical regions; for example, mycotoxins such as aflatoxins occur more frequently in tropical and subtropical climates (Leslie *et al.*2008; Mannaa and Kim, 2017).Because of their chemical resistance,

removing fungus from food does not ensure the absence of mycotoxins (Perdoncini *et al.* 2019). Because of their frequent contamination of nourishment meant for human and animal consumption and the extraordinarily severe toxicological effects they inflict on the mammalian system upon ingestion, aflatoxins are the most significant type of fungal toxin (Dubey, 2013).The most well researched toxins, aflatoxins, are among the most carcinogenic substances known and continue to be the most important mycotoxin. *Aspergillus flavus* and *Aspergillus parasiticus* are the primary producers of aflatoxin, a class of highly hazardous secondary metabolites. Aflatoxin is a heterocyclic molecule with high oxygen content.The eight identified aflatoxins are B1, B2, G1, G2 (called by their blue or green fluorescence in UV light), M1, M2 (initially recognised in milk of cows fed groundnut meal), and B2a and G2a (derivatives of B2 and G2). These may found independently or occurs together. The different derivatives differ in their toxicity. Among all aflatoxin B1 is most commonly found and most toxic in nature. The presence of furfuran ring is essential for toxic and carcinogenic activity of aflatoxin. Aflatoxin is a serious hazard to both animal production and human health, resulting in massive global economic losses each year.The aflatoxin B1 is one of the most important factor responsible for liver cancer in several population of South-East Asia and Africa.

Aflatoxin contamination is the most serious food safety issue for field crops grown in tropical and subtropical climates where high temperatures and humidity favour the establishment and proliferation of *Aspergillus* spp. Rice, oil seeds, almonds, dried fruit, spices, and beans are among the major food items affected (Reddy *et al.* 2009).Their melting points are over 250 degrees Celsius, and they are stable at pH levels ranging from 3 to 10 (Ruadrew *et al*. 2013). Due to their stability and thermal resistance in dried products, AFS removal is extremely difficult (Lee *et al.*2015). AFS is resistant to food processing; therefore it may persist throughout the food chain. As a result, AFS are potential hazards to human health, either through direct ingestion of contaminated food products or by the transfer of aflatoxins and their metabolites in milk and meat (Naseer *et al.*2014). AFB1 is the most hazardous, and the International Agency for Research on Cancer (IARC) has classed it as a Group I carcinogen. Epidemiological studies have linked it to liver cancer and acute hepatitis (Ruadrew *et al.*2013).Unfortunately, dietary AFS is a chronic problem in tropical regions and around the world due to increased global trade and food transportation across borders.More than a hundred nations have created laws to reduce AFS content in agricultural commodities in order to manage this safety issue(Reddy *et al*. 2009).The present investigation focuses on the production of Aflatoxin B1 from a standard toxicogenic strain of *A. flavus* in different medium and comparative yield analysis with the help of HPLC-FLD.

MATERIALS AND METHODS

Chemical and reagents

All the media ingredients of CYA (Czapek's Yeast Extract Agar), SKMY(200 g sucrose, 0.5 g magnesium sulphate, 3 g potassium nitrate, 7 g yeast extract and 1000 mL of distilled water) were purchased from Sigma-Aldrich. The entire chemical reagents bought are of HPLC grade. Aflatoxin standard were purchased from R-Biopharm Neugen Pvt. Ltd., Hyderabad, India, in a mixture form (concentrations are as follow, AFB_1-1 µg/ml, AFB_2-0.5 µg/ml, AFG_{1-} 1 µg/ml and AFG_{2-} 0.5 µg/ml) of 2ml. The standard was stored at 4^0C away from light before use.

Source of toxicogenic fungal strain

Aflatoxin producing strain of *Aspergillus flavus* MTCC 2798 obtained from Microbial Type Culture Collection, Chandigarh, India. The fungal strain was propagated on CYA slants and the morphology and microscopic features of the culture were noted. Pure culture was maintained at 4^0C until use.

Detection of aflatoxin in SKMY culture condition

The spores of *A.flavus* MTCC 2798 were aseptically taken with the help of 1µl inoculating loop and transferred into different broth medium in triplicates of SKMY and incubated under static condition at

room temperature for 15 days. In SKMY medium mold broth filtered through normal filter paper followed by Whatman No.1 filter paper and the filtrate was collected in the flask. In this filtrate equal volume of chloroform was added and kept in shaking condition for 30 minutes. The chloroform fraction was collected through separating funnel and then it was passed through sodium sulphate column to remove the moisture content. This fraction were subjected to thin layer chromatography containing TLC Silica gel 60 F_{254} plate 20 cm X 20 cm (Merck, Dermstadt, Germany) as stationary phase and a mixture of ether-methanol-water (96:3:1,v/v) as the mobile phase. Aflatoxin B1 is confirmed by the presence of fluorescence band having Rf value same as of standard visualized under UV light using a UV-transilluminator.

Extraction and partition of aflatoxin from rice and coffee medium

For growth of toxicogenic strain the rice and coffee medium of 35% moisture content were taken. After the growth of mycelia for 15 days at room temperature, the mycelial broth was taken into a blender jar and extract with methanol:water (60:40) was added and the contents were blended for 3 min at high speed and filtered through Whatman No.1 filter paper. This filtrate was collected and 9ml of saturated NaCl along with 15 ml of hexane was added and shaken for 5 minutes. Now this mixture was added to separating funnel and the lower aqueous phase was taken into another separating funnel. To this 10ml of chloroform was added and then take the chloroform layer in another separating funnel to this 1.8g cupric carbonate was added and again it was separated through separating funnel followed by this it was passed through bed of sodium sulphate for removal of any moisture content. This resulting fraction was evaporated for 2-3 ml in hot air oven at 40^0C.

Quantification of aflatoxin using High-PerformanceLiquid Chromatography with fluorescence detector (HPLC-FLD)

Aflatoxin extraction with immuno-affinity clean-up

The dried chloroform extract were mixed with PBS buffer. Immunoaffinity column (IAC, R-Biopharm Rhône Ltd., Glasgow, UK) were used for sample purification and AFS extraction. Diluted filtrate (20 ml) was passed through IAC at approximately 0.6 ml/min by gravity. A slow flow rate was necessary for capturing AFS by their antibodies in IAC, as recommended by the manufacturer.Then IAC was dried by passing air (syringe) for 10s. AFS were eluted from IAC by 0.5 ml methanol then 0.5 ml DDW accompanied by back flushing. Air was passed through the column using a syringe to collect the last few drops. Collected elutes were clarified by a disposable filter membrane (sterile 0.45 mm) and stored in autosampler amber vials (Agilent Technology) at 4^0C prior to injection into HPLC system.

Detection of aflatoxin of HPLC-FLD

A reversed phased (RP) HPLC procedure was used for AFS determination in sample.The method described by (Zhu *et al.*2013) was followed with few modifications. A 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used for quantification of aflatoxins.The isocratic mobile phase was a mixture of water: methanol (60:40, v/v) with a flow rate of 1 ml/min. Level of detections (LOD) of AFB1, AFB2, AFG1, and AFG2 were established by practical experimentation as being 0.005, 0.005, 0.005, and 0.005 mg/kg, respectively. Filtered samples (20 µl) were injected into system through the autosampler and AFS signals were detected with FLD at excitation of 365 nm and 435 nm emission. The data of three replicates were acquired and analyzed with the Agilent Data Handling Chemstation 3.

Spiking experiment

For accuracy of the experiment, spike analyses of the negative control fungal strain extract were spiked at 2 different levels of 2 and 5 ppb. The spiked samples were analyzed for recovery.

RESULT AND DISCUSSION

Aflatoxin producing fungal strain

The toxicogenic strain MTCC 2798 obtained from MTCC, Chandigarh, India. This strain was

Table 1: Recovery(%) of aflatoxins(AFS) from samples spiked with two different concentrations

	Aflatoxin concentration (µg/kg)					
	level-1	obtained	% recovery	level-2	obtained	% recovery
Aflatoxin B1	2	2.03	101.5	5	5.04	100.8
Aflatoxin B2	2	1.98	99	5	4.99	99.8
Aflatoxin G1	2	2.01	100.5	5	5.02	100.4
Aflatoxin G2	2	1.99	99.5	5	4.96	99.2
Aflatoxins	8	8.01	100.125	20	20.01	100.05

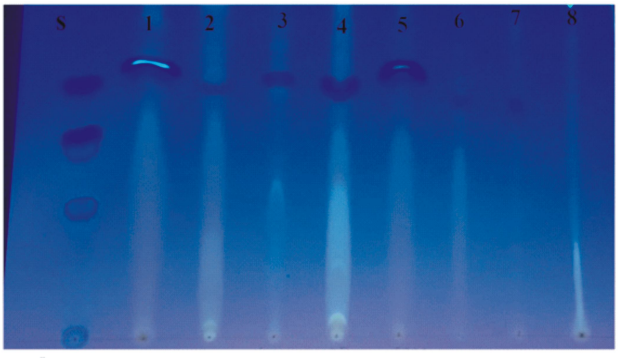

Fig.1: Representative TLC image at long wave UV light of aflatoxin standard 2 ppb in S lane of 50 µl volume; 2798 strain extract in chloroform 50 µl volume in lane 1; 2798 strain extract in chloroform 10 µl volume in lane 2; 2798 strain extract in chloroform 20 µl volume in lane 3; 2798 strain extract in chloroform 30 µl volume in lane 4;2798 strain extract in chloroform 40 µl volume in lane 5; lane 6, lane 7 and lane 8 contain negative control.

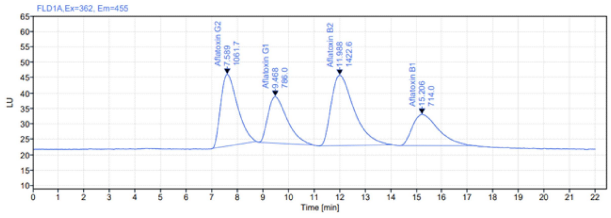

Fig.2: Representative chromatogram of aflatoxin standard 5ppb

propogated on CYA media, which produced green powdery colonies on the plate. Microscopic characterization was done on 10x, 40x and 100x. It shows presence of conidia in the form of globose vesicles at the end of conidiophores.

Detection of aflatoxin production

Primary detection was done for aflatoxin production on synthetic media SKMY broth and it was incubated for 15 days at room temperature in static condition. As the incubation time increases after 7 days, the medium developed a yellowish white colony; this is due to production of secondary metabolites. As the fungal growth approaches to 15 day, it was extracted with chloroform and then subjected to thin layer chromatography for qualitative detection of it. The TLC plate shows

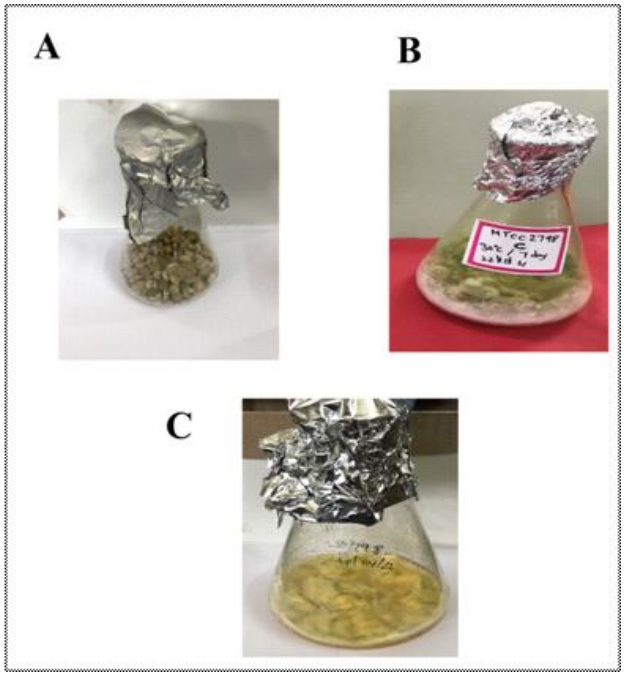

Fig.3: Aflatoxin production in different medium: A - represents aflatoxin production in coffee medium; B - represents aflatoxin production in rice medium; C - represents aflatoxin production in SKMY medium.

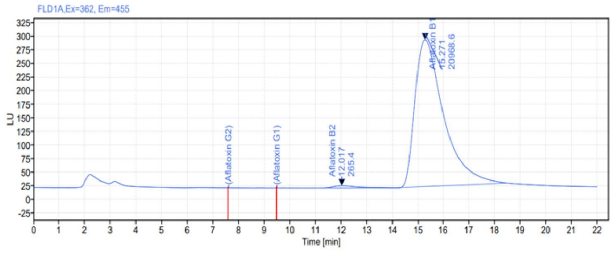

Fig.4: Representative chromatogram of aflatoxin production of 2798 in rice medium

presence of blue fluorescence in UV-transillumiator at long UV wave, which indicates presence of aflatoxin B in the sample represented in Fig. 1.

Aflatoxin recovery by HPLC-FLD

Spiked sample analyses are used to correct the actual concentration of an analyte (e.g. Aflatoxin) in samples (Trucksess *et al*. 2011). Fig.2 shows HPLC chromatogram and elution times of Aflatoxins standards. Their order is AFG2 (7.589 min), AFG1 (9.468 min), AFB2 (11.988 min) and AFB1 (15.206 min). Recoveries of AFS form spiked sample (blank) are as shown in (Table 1)**.** Total Aflatoxin recovery was 100.125% and 100.05% at spiked concentrations of 2 and 5 µg/kg, respectively. Those percentages were within acceptable values for AFS of the AOAC, Codex Alimentarius and EU Commission which shows acceptable range between 70-120%.The LOD for analysis was found to be 0.3µg/kg and LOQ was 0.5µg/kg.

Quantification of aflatoxin in rice, coffee and SKMY medium

In present study, visible growth of MTCC strain 2798 *A. flavus* in rice grain medium and coffee medium with 35% moisture content was denser at 15 days as compared 7 days represented in Fig.3. Mycelial growth at 15 day grown more deeper green color as compared to day 7. *A. flavus* formed only AFB1 and AFB2 at room temperature. AFG1 and AFG2 were not detected at room temperature throughout the storage period. The aflatoxin production detected by HPLC-FLD was shown presence of high amount of aflatoxin in rice medium as compared to coffee medium and synthetic media SKMY. The amount of aflatoxin in rice medium was found to be 455 µg/kg represented in Fig.4, in coffee medium it was found to be 381 µg/kg and in SKMY it was found to be 122 µg/kg.

CONCLUSION

This study suggests the effect of different media on production and quantification of aflatoxin. The level of toxin production varies between synthetic and natural media. The natural media for production of toxin are cost effective and easy to dispose as compared to synthetic media.

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