Screening of Aflatoxin Production by *Aspergillus flavus* Isolates from Petroleum-contaminated Soil

Sawan M. Mirkhan and Taha J. Zrary

Department of Biology, Faculty of Science and Health, Koya University,
Danielle Mitterrand Boulevard, Koya KOY45, Kurdistan Region – F.R. Iraq

***Abstract*—*Fungi* are eukaryotic, heterogeneous, unicellular to filamentous, spore-bearing, and chemoorganotrophic organisms which lack chlorophyll. This present study was carried out to isolate and identify fungi from petroleum-contaminated soil. Several fungal genera which included *Rhizopus* spp., *Mucor* spp., *Penicillium* spp., *Rhizoctonia* spp., *Aspergillus* spp., *Alternaria* spp., and *Cladosporium* spp. were isolated using potatoes dextrose agar, Czapek-Dox Agar, and *Aspergillus flavus* Differentiation Agar culture media that comparable with co-amoxiclav (1g) and chloramphenicol to prevent the growth of any bacteria. The direct plate and serial dilution agar plate methods were used for the isolation of fungi. Based on the results, *Aspergillus* and *Mucor* spp. were the most predominant genera and had the highest number of colonies in the soil samples. In this investigation, seven out of 27 soil samples were morphologically (macroscopically and microscopically) identified, such as *A. flavus*. Aflatoxigenicity of *A. flavus* was detected using characteristics in Aspergillus differentiation agar and colony fluorescence on exposure to ultraviolet light. In addition, molecular approaches were used for the detection of aflatoxigenic of the *A. flavus* isolates. Three structural (*aflD, aflO,* and *aflP*) and one regulatory (*aflR*) gene of the aflatoxin gene cluster of *A. flavus* were targeted for amplification by the polymerase chain reaction method. The aflatoxigenic of all six *A. flavus* isolates was detected molecularly which contained two structural *(aflD, aflP)* genes out of three structural genes, while there was no specific amplification of the *aflO* gene in the fourth, fifth, and sixth *A. flavus* which is similar to the aflR gene in the first and second *A. flavus*.**

***Index Terms*—Fungus, Aflatoxin, Aflatoxin genes, *Aspergillus flavus*, Petroleum-contaminated soil, Polymerase chain reaction.**

I. Introduction

Fungi do not photosynthesize; therefore, they get their food by the absorption of dissolved molecules due to their ability to secret some digestive enzymes to their environment as they are heterotrophs. Thus, they are considered to be the main decomposers in ecological systems (Harris, 2008; Soni, 2007).

Microfungi contribute to the degradation of synthetic and natural pollutants because fungi similarly emit primary and secondary metabolites (antibiotics, protein, toxins, ethanol, etc.). Soil fungi are a potential and abundant source of highly bioactive secondary metabolites. Mycoflora colonizes, reproduces, and stays alive in several environments including microecological niches. They can survive in risky environments, such as thermal springs, cold places, hot springs, and run-off hydrocarbon-contaminated soil (Hanson, 2008).

Mycotoxins are non-volatile, and they are mainly produced by filamentous fungi; however, it should be taken into consideration that the majority of fungi are non-toxigenic and many secondary metabolites from fungi are non-toxic. Mycotoxins occur in varieties of fungi, some of these mycotoxins are important and intensive, together with ochratoxin, citrinin, ergot alkaloid, patulin, fusarium, and aflatoxin. Aflatoxins are potent carcinogenic, mutagenic, and teratogenic secondary metabolites and are produced predominantly by *Aspergillus flavus* and *Aspergillus parasiticus* (Abdel-Hadi, 2011). *A. flavus* is a saprotrophic and pathogenic fungus with a cosmopolitan distribution (Ramírez-Camejo, et al., 2012).

Petroleum-based products are complex mixtures that contain numerous aliphatic, alicyclic, and aromatic compounds depending on the definition. It is well known as a great important source of daily energy during the exploration, production, refining, transport, storage of petroleum, and petroleum products (Kvenvolden and Cooper, 2003).

This saprotrophic *A. flavus* can survive on most of the sources of organic nutrients, such as tree leaves, animal fodder, outdoor and indoor air environments, dead insects and animal carcasses, and etcetera; therefore it is so common. It is a pathogenic fungus with a cosmopolitan distribution (Ramírez-Camejo, et al., 2012; Machida and Gomi, 2010).

Aflatoxin is a type of mycotoxin produced by *Aspergillus* species of fungi, such as *A. flavus* and *A. parasiticus* (Martins, Martins and Bernardo, 2001). Aflatoxins consist of a group of approximately 20 related fungal metabolites. Yin, et al. (2008) declared that the term aflatoxin comes from four different types of the production of mycotoxins, which are B_1, B_2, G_1, and G_2. Aflatoxin B_1 is the most toxic and potent carcinogen that can cause liver cancer in many different

ARO-The Scientific Journal of Koya University
Vol. XI, No. 2 (2023), Article ID: ARO.11144. 6 pages
DOI: 10.14500/aro.11144
Received: 01 February 2023; Accepted: 28 July 2023
Regular research paper: Published: 25 August 2023
Corresponding author's e-mail: sawan.majid@koyauniversity.org

animal species (Martins, Martins and Bernardo, 2001). Besides, it contributes to the production of the tropics and subtropics commodities, such as pistachios, cotton, spices, and maize (Yin, et al., 2008; Martins, Martins and Bernardo, 2001).

II. Materials and Methods

A. Scanning Location

Twenty-seven soil samples were collected from different ecosystems at Hawler governorate (Taq Taq Operating Company) and Sulaymaniyah governorate (Bazian Oil Refinery and Directory Refinery of Sulaymaniah) that were contaminated with petroleum. Where the samples of soil were taken from, growing plants were noticed. All samples were collected from the 2nd of December 2015 to the 10th of March 2016.

B. Soil Sampling

Soil samples (about 500 g each) were taken from a depth of 0–15 cm from petroleum-contaminated soil in each refinery field. The distance between every two samples was 25 m; however, three of them were taken randomly (as replication) at three different points with a trowel after removing litter or weed plants.

C. Morphological Identification of Aspergillus Species

Aspergillus isolates were grown on potatoes dextrose agar (PDA), Czapek-Dox Agar (CZA), and AFPA at 28°C incubation to identify macroscopic characters including color, diameter, and number of colonies, and microscopic characters, such as conidia, conidiophore, phialides, and vesicles. After that, slides were prepared from these new cultures using Lacto phenol cotton blue by growing medium and then observed under a light microscope (Rodrigues, et al., 2007).

D. Cultural Methods for Aflatoxin Detection

Detection of aflatoxin based on cultural characteristics in AFPA

For the aflatoxin detection that creates *Aspergillus* species from soil samples, Aspergillus differentiation agar was used, which is a differential and selective medium. Then, aflatoxigenic *A. flavus* was distinguished after 7 days of incubation at 28 °C depending on the appearance of the reverse orange color on the plates. The cause of this color change is the reaction between the aspergillic acid molecules that are produced by *Aspergillus* species with the ferric ions from ferric citrate, which exists in the medium (Rodrigues, et al., 2007).

E. Molecular Identification of A. flavus Isolates

Classical techniques were used for the identification and detection of *Aspergillus* isolates in samples based on morphological studies. These methods have a low degree of sensitivity and do not allow the specification of mycotoxigenic species. PCR methods are used to detect aflatoxigenic strains of *A. flavus,* as they can target DNA accurately due to their high sensitivity and specificity (Almoammar, et al., 2013).

F. DNA Extraction Method

DNA extraction kit (Fungal/Bacterial DNA MiniPrep™) is used to extract the genomic DNA from all the isolates. The kit is used according to the manufacturer.

G. Molecular Discovery of Aflatoxigenic A. flavus Isolates

Aflatoxigenic strains of *A. flavus* was distinguished and identified from other fungi by developing Molecular methods. A polymerase chain reaction (PCR) is one of those techniques that are used for this purpose as it can be handy and easy to use (Hussain, et al., 2015; Criseo, Bagnara and Bisignano, 2001). Cluster genes contain *nor1, omtB,* and *omt-1* structural genes in the aflatoxin biosynthesis pathway, which is coding for key enzymes and regulatory gene (*aflR*) which is vital for aflatoxin production; besides, it affects the structural genes and activates transcription. Four primer pairs have been used for a specific consequence of *aflO, aflD, aflR, and aflP* (Erami, et al., 2007). Numerous research assemblies have tested the possibility of applying PCR-based diagnostic methods to detect the presence (genomic PCR) or real-time PCR of the aflatoxin biosynthetic genes or the expression reverse transcription (RT) (Dheeb, et al., 2014; Abdel-Hadi, Carter and Magan, 2011; Degola, et al., 2007) (Tables I-IV).

The PCR products were detected on 2% agarose at 5 volts/cm^2. 1x TBE buffer for 1:30 h, DNA ladder (100 bp), visualized under U.V light Agreed by (Scherm, et al., 2005).

H. Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) Version 17.0. Results are expressed as mean ± S.E. The analysis of variance (ANOVA) was applied.

TABLE I
The Components of the Maxime PCR PreMix kit (i-Taq)

Materials	Volume
i-Taq DNA polymerase (U)	2.5
DNTPs (mm)	2.5
Reaction buffer (10X)	1X
Gel loading buffer	1X

PCR: Polymerase chain reaction

TABLE II
Mixture of the Specific Interaction for Diagnosis Gene

Components	Concentration
Maxime PCR PreMix kit (i-Taq) (μL)	5
Forward primer	10 pmol/μ (1 μL)
Reverse primer	10 pmol/μ (1 μL)
DNA (μL)	1–1.5
Distill water (μL)	16.5
Final volume (μL)	25

PCR: Polymerase chain reaction

TABLE III
PRIMERS THAT ARE USED (SCHERM, ET AL., 2005)

Gene	Primer code	Sequence (5'–3')	Temperature (°C)	GC (%)	Estimated size (bp)
aflD	Nor1 (F)	ACGGATCACTTAGCCAGCAC	57.3	55.0	990
	Nor1 (R)	CTACCAGGGGAGTTGAGATCC	56.4	57.1	
AflO	OmtB (F)	GCCTTGACATGGAAACCATC	54.1	50.0	1333
	OmtB (R)	CCAAGATGGCCTGCTCTTTA	54.9	50.0	
AflP	Omt1 (F)	GCCTTGCAAACACACTTTCA	54.4	54.0	1490
	Omt1 (R)	AGTTGTTGAACGCCCCAGT	57.3	52.6	
aflR	AflR (F)	CGAGTTGTGCCAGTTCAAAA	53.8	45.0	999
	AflR (R)	AATCCTCGCCCACCATACTA	57.3	52.6	

III. RESULTS

A. Isolation of Fungi

Direct method

Approximately 27 soil samples were taken from three different refineries from December 2015 to March 2016. The samples were cultured on PDA with co-amoxiclav (1g), CZA with co-amoxiclav (1g), and *A. flavus* Differentiation Agar (AFDA) with chloramphenicol (Table V).

B. Morphological Identification of A. flavus

Aspergillus has several morphological and growth responses to different nutrients, so it is significant to regulate conditions for the morphological detection of *A. flavus*, as species identification depends on pure cultures grown on known media.

The plates were incubated for 7 days at 28ºC on CZA and PDA, and then, they were observed for macroscopic (colony color and diameter) (Fig. 1) and microscopic characteristics (conidia, conidiophore, vesicle, and phialides) (Figs. 2 and 3).

C. Cultural Method for Detection of Aflatoxin

There are several methods for the detection of aflatoxin in cultures of fungus. Here, the colony fluorescence on exposure to ultraviolet (UV) and orange color features in AFPA was used (Yazdani, et al., 2010).

Detection of aflatoxin producers based on reverse color change on AFDA

Aflatoxin-producing *Aspergillus* species were detected on Aspergillus differentiation agar (AFPA), which is a selective and differential medium. In Fig. 4a, a white colony can be seen after 7 days of incubation at 28°C, while in Fig. 4b, an orange colony can be seen on the reverse side of the plate.

D. Molecular Identification of A. flavus

Extraction of DNA

Six out of 27 *A. flavus* isolates were identified by KAPA Universal Ladder and contain four reference bands (500, 1000, 1600, and 4000) for orientation, (Fig. 5).

Detection of aflatoxin-producing strains by conventional PCR method

PCR was used to diagnose aflatoxin-making strains of *A. flavus* molecularly, which are three structural genes (aflD, aflO, and aflP), and a regulatory gene (*aflR).* The results of the toxigenicity of all *A. flavus* isolates have shown that only *aflD* and *aflP* among the structural genes and *aflR* as a regulatory gene were identified as the most toxigenic isolates (Fig. 6).

TABLE IV
THE OPTIMUM CONDITION OF THE DETECTION GENE (SCHERM, ET AL., 2005)

Phase	Temperature (°C)	Time	Number of cycle
Initial denaturation	94	5 min	35 cycles
Denaturation 2	94	30 s	
Annealing	50	60 s	
Extension	72	90 s	
Final extension	72	7 min	

TABLE V
THE INCIDENCE PERCENTAGE OF FUNGI ISOLATED FROM CONTAMINATED SOIL BY THE DIRECT METHOD

Isolated fungi	Frequency (%)	Location
Rhizopus spp.	2 (6.9)	Bazyan
Mucor spp.	7 (24.1)	Bazyan, DRS, TTOPCO
Penicillium spp.	3 (10.4)	Bazyan, DRS, TTOPCO
Rhizoctonia spp.	1 (3.5)	Bazyan
Aspergillus spp.	7 (24.1)	DRS, TTOPCO
Alternaria spp.	6 (20.7)	DRS, TTOPCO
Cladosporium spp.	3 (10.3)	DRS
Total	29 (100)	

DRS: Directory Refinery of Sulaymaniyah, TTOPCO: Taq Taq Operating Company

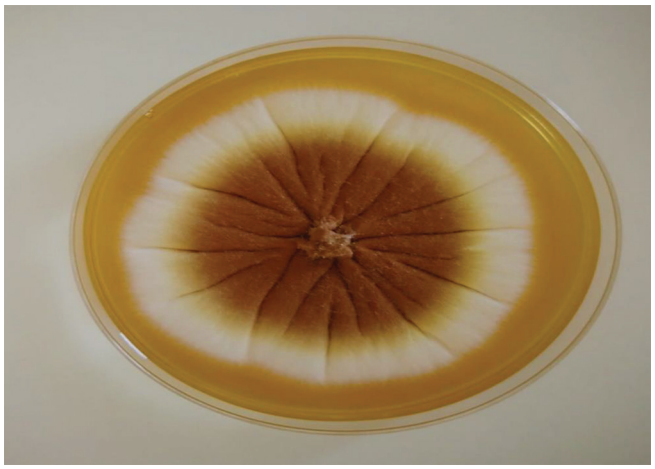

Fig. 1. Colony of *Aspergillus flavus* on CZA attaining diameter 36 mm within 7 days of incubation at 28ºC, colony color becoming brown to white.

http://dx.doi.org/10.14500/aro.11144

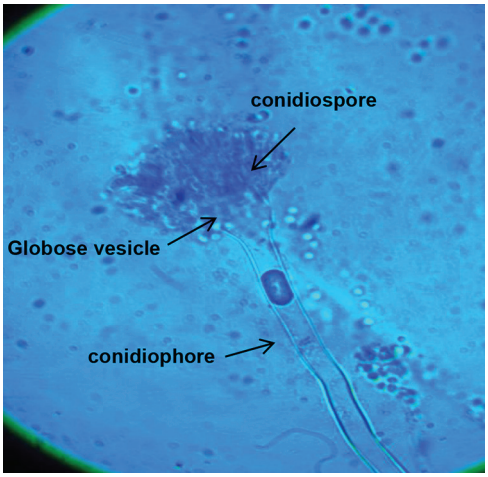

Fig. 2. Microscopic characteristic of *Aspergillus flavus*, Aspergillum-like spore-bearing, globose vesicle, bear chain of conidiospores; microscopic observation of the fungal isolate under ×400 magnification (lactophenol cotton).

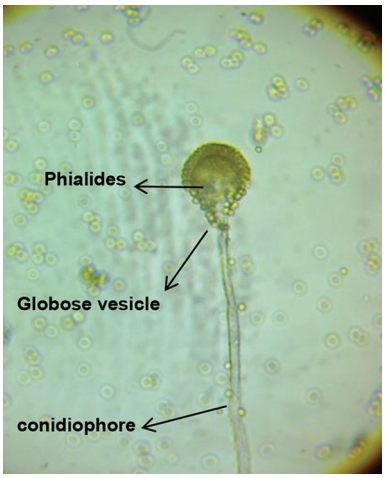

Fig. 3. Uniseriate Conidia head of *Aspergillus favus,* Aspergillum-like spore-bearing conidiophore, Globose vesicle, directly borne vesicles. Microscopic observation of the fungal isolate under ×400.

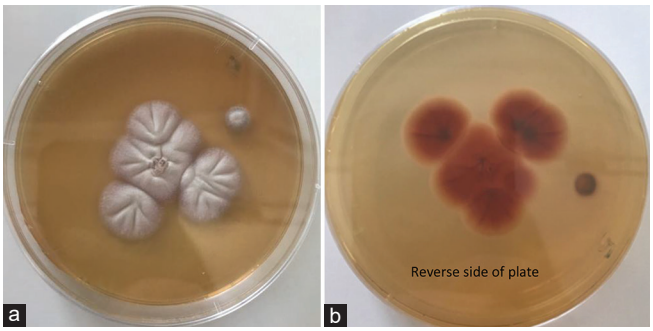

Fig. 4. (a and b) *Aspergillus flavus* on AFPA, after 7 days of incubation at 28°C, with characteristic orange color on the reverse side of the plate.

IV. Discussion

In the investigation, seven isolated genera were obtained from the 27 soil samples that were isolated from various locations. From the fungal isolates, most of the species belonging to the genera *Aspergillus* and *Mucor* were

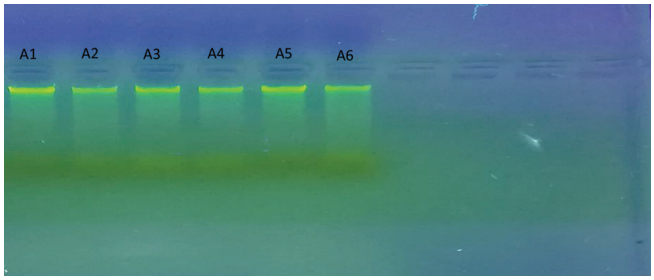

Fig. 5. Gel electrophoresis of genomic DNA extraction from *Aspergillus flavus*, 1% agarose gel at 5 vol/cm for 1:15 h, A: *A. flavus*.

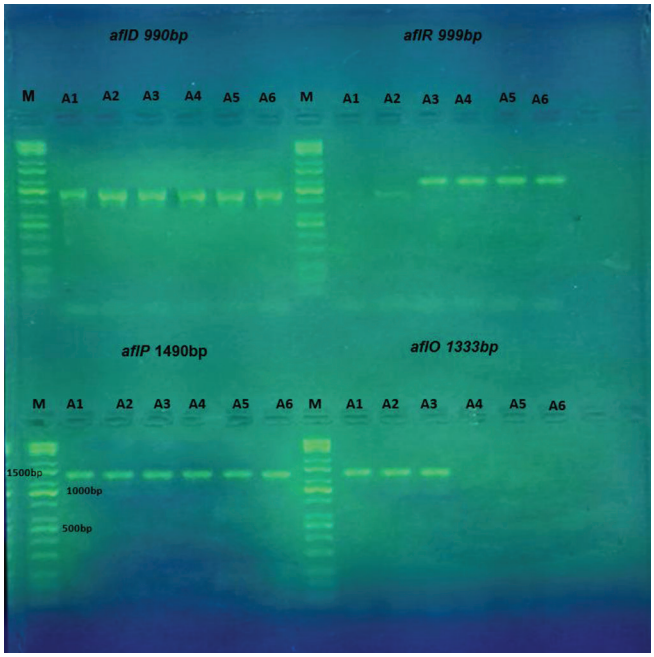

Fig. 6. The polymerase chain reaction product of the band size (*aflD* 990 pb) (*aflO* 1333 bp) (*aflP* 1490 bp) (*aflR*1498 bp). The product was electrophoresis on 2% agarose at 5 volt/cm^2. ×1 TBE buffer for 1:30 h. N: DNA ladder (100), lane (1–6), visualized under UV light, A: *Aspergillus flavus*.

dominant. The same results were achieved by Raja, Praveena and William in 2017. These results were achieved with those found by Rohilla and Salar (2012) as well. 24.1% of *Aspergillus* spp. and 20.7% of *Alternaria* spp. were found in the Directory Refinery of Sulaymaniyah (DRS), and Taq Taq Operating Company (TTOPCO), while 24.1% of *Mucor* spp. and 10.1% of *Penicillium* spp. found in DRS, TTOPCO, and Bazyan. Finally, *Rhizoctonia* spp., *Rhizopus* spp., and *Cladosporium* spp. from Bazyan were 3.5%, 6.9%, and 10.3%, respectively. These results correspond with Raja, Praveena and William in 2017.

Results from the present study show a correlation between dilution with the diameter and the number of colonies of isolate fungi in different soil contaminated with petroleum locations which were reported by Farrag, et al. (2017); Agarwal (2017), and Jasuja, Saxena and Joshi (2013).

Possibly, due to differences in the cell wall structure, fungi are considered to be more tolerant of high concentrations of polluting chemicals than other organisms. In fungi, the

cell wall is the outer-most layer, external to the plasma membrane. The fungal cell wall is a matrix of three main components chitin, glucans, and proteins (Blakely, et al., 2002). Based on Khalid, Hussain and Imran (2018); Farrag, et al. (2017), and Giusiano, et al. (2017), all *A. flavus* isolates were identified depending on the features' description and the isolates' characteristics. *A. flavus* is morphologically characterized by brown to white smooth colonies on CZA. As stated by Hedayati, et al., (2007), the colonies were flat, with radial grooves. It was observed that the diameter of *A. flavus* colonies was smaller on CZA, PDA, and CMA than on AFPA. This difference in the diameter of the colony of fungi may have been caused by the difference in the nutrient ingredients of the media.

To differentiate aflatoxigenic from non-aflatoxigenic Aspergillus, three different cultural-based methods were used. These were the blue fluorescence of aflatoxin B, orange color on the reverse of the AFPA plates, and color change on exposure to ammonium hydroxide vapor which agreed with Yazdani, et al. (2010) and Rodrigues, et al. (2007). However, in 2014, Nair, et al. demonstrated that mycotoxins cannot be produced by all strains of *Aspergillus*, as *A. flavus* producing aflatoxin needs using techniques such as ELISA, TLC, and HPLC to obtain aflatoxin concentration.

In this study, seven *Aspergillus* spp. were isolated out of 27 samples, while six samples of *A. flavus* were used for the molecular method by correlating between the presence-absence of genes and also the ability-inability of aflatoxins production. Nowadays, to identify and detect aflatoxin-producing fungi, DNA-based detection systems are frequently used, such as PCR, which is used to distinguish between the aflatoxin-producing and non-producing *A. flavus* strains (Hussain, et al., 2015; Degola, et al., 2007). In 2016, Temu declared that there are three structural genes (*aflD, aflM,* and *aflO)* with aflR as a regulatory gene that was followed by 5.8S ITS rDNA nucleotide sequence analysis; besides, the spices quality with safety prediction to consumers can be assessed by PCR. The presence of fungi in a substrate does not necessarily relate to the presence or amount of aflatoxin, however, bands of the fragments of *aflD* and *aflP* genes at 990 bp and 1490 bp were visualized, respectively, as the result of the test for the detection of aflatoxigenic *A. flavus* isolates using the molecular method. While *aflR* was seen frequently in *A. flavus* samples in our condition and even with using the same pair of primers, aflO was seen in three *A. flavus* and not visualized in other *Aspergillus* strains, as shown in Fig. 6. The lack of aflatoxin production in the strains of *A. flavus* may be due to the lack of genes in their genome, as reported by Houshyarfard, et al. (2014). Deletion of portions of the aflatoxin biosynthesis gene cluster within the aflatoxigenic *A. flavus* strain is not rare. In 2016, Temu stated that the genetic variation in aflatoxin production can arise from mutations in the aflatoxin biosynthetic gene cluster which may include gene loss, DNA inversion, recombination, partial deletions, or other genomic rearrangements of the cluster (Carbone, et al., 2007). However, a species may have all genes involved in the pathway but not necessarily produce aflatoxin till certain favorable conditions trigger its production. However, a species missing aflatoxin biosynthetic genes cannot produce aflatoxin. The presence of biosynthetic genes has been used as a diagnostic tool for aflatoxigenic fungi in certain foodstuffs (Gallo, et al., 2012).

V. Conclusions

From this presented study, we have concluded that the most frequent fungi isolates were *Aspergillus spp*. and *Mucor spp.*; however, the amplification of *aflD* and *aflP* genes from *A. flavus* isolates from the petroleum-contaminated could confirm the aflatoxin production. Hence, not necessarily all *Aspergillus spp*. excrete aflatoxin (note: all the structural aflatoxin genes were not detected in current local isolates of *A. flavus*).

References

Abdel-Hadi, A., 2011. Molecular Ecology of *Aspergillus* Section Flavi Species: Approaches to Understanding the Role of Aflatoxin Genes in Aflatoxin Biosynthesis.

Abdel-Hadi, A., Carter, D., and Magan, N., 2011. Discrimination between aflatoxigenic and non-aflatoxigenic *Aspergillus* section Flavi strains from Egyptian peanuts using molecular and analytical techniques. *World Mycotoxin Journal*, 4(1), pp.69-77.

Agarwal, P., 2017. Isolation and characterization of Tyrosinase (a carbon trapping enzyme) producing microorganisms, in the agricultural soil of Western Uttar Pradesh and the study of enzymatic activity of Tyrosinase produced. *Biochemistry and Molecular Biology Letters*, 3(1), p.105.

Almoammar, H., Bahkali, A.H., and Abd-Elsalam, K.A., 2013. A polyphasic method for the identification of aflatoxigenic '*Aspergillus*' species isolated from Camel feeds. *Australian Journal of Crop Science*, 7(11), p.1707.

Blakely, J.K., Neher, D.A. and Spongberg, A.L., 2002. Soil invertebrate and microbial communities, and decomposition as indicators of polycyclic aromatic hydrocarbon contamination. *Applied Soil Ecology*, 21(1), p.71-88.

Carbone, I., Jakobek, J.L., Ramirez-Prado, J.H., and Horn, B.W., 2007. Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. *Molecular Ecology*, 16(20), pp.4401-4417.

Criseo, G., Bagnara, A., and Bisignano, G., 2001. Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. *Letters in Applied Microbiology*, 33(4), pp.291-295.

Degola, F., Berni, E., Dall'Asta, C., Spotti, E., Marchelli, R., Ferrero, I., and Restivo, F.M., 2007. A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. *Journal of Applied Microbiology*, 103(2), pp.409-417.

Dheeb, B.I., Ismail, E.N., AL-mishhadani, I.I.H., Majeed, S.M., and Majeed, D.M., 2014. A study of the expression of aflatoxin B1 regulatory gene in clinical and environmental *Aspergillus flavus* using real-time PCR. *International Journal of Sciences: Basic and Applied Research*, 17(1), pp.417-427.

Erami, M., Hashemi, S.J., Pourbakhsh, S.A., Shahsavandi, S., Mohammadi, S., Shoushtari, A., and Jahan, S.Z., 2007. Application of PCR on detection of aflatoxinogenic fungi; short communication. *Archives of Razi Institute*, 62, pp.95-100.

Farrag, A.A., Shehata, R.M., EL-Sheikh, H.H., Abo-Dahab, N.F., and Ali, A.M., 2017. Diversity and biotechnological applications of some fungi that isolated from unusual soil samples in Egypt. *Journal of Ecology of Health and Environment*, 5(1), pp.23-33.

Gallo, A., Stea, G., Battilani, P., Logrieco, A.F., and Perrone, G., 2012. Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy. *Phytopathologia*

Mediterranea, 51, pp.198-206.

Giusiano, G.E., Piontelli, E., Fernández, M.S., Mangiaterra, M.L., Cattana, M.E., Kocsubé, S., and Varga, J., 2017. Biodiversity of species of *Aspergillus* section Fumigati in semi-desert soils in Argentina. *Revista Argentina de microbiologia*, 49(3), pp.247-254.

Hanson, J.R., 2008. *Chemistry of Fungi*. Royal Society of Chemistry, United Kingdom.

Harris, S.D., 2008. Branching of fungal hyphae: Regulation, mechanisms and comparison with other branching systems. *Mycologia*, 100(6), pp.823-832.

Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., and Denning, D.W., 2007. *Aspergillus flavus*: Human pathogen, allergen and mycotoxin producer. *Microbiology*, 153(6), pp.1677-1692.

Houshyarfard, M., Rouhani, H., Falahati-Rastegar, M., Malekzadeh-Shafaroudi, S., Mehdikhani-Moghaddam, E., and Chang, P.K., 2014. Gene deletion patterns in non-aflatoxigenic isolates of *Aspergillus flavus*. *Mycologia Iranica*, 1(2), pp.87-97.

Hussain, A., Afzal, A., Irfan, M., and Malik, K.A., 2015. Molecular detection of aflatoxin-producing strains of *Aspergillus flavus* from peanut (*Arachis hypogaea*). *Turkish Journal of Agriculture-Food Science and Technology*, 3(5), pp.335-341.

Jasuja, N.D., Saxena, R., and Joshi, S.C., 2013. Isolation and identification of microorganisms from playhouse agriculture soil of Rajasthan. *African Journal of Microbiology Research*, 7(41), pp.4886-4891.

Khalid, S., Hussain, N., and Imran, M., 2018. Detection of aflatoxigenicity of *Aspergillus flavus*, based on potential gene marker, from food and feed samples. *Journal of Food Safety*, 38, p.e12448.

Kvenvolden, K.A., and Cooper, C.K., 2003. Natural seepage of crude oil into the marine environment. *Geo-Marine Letters*, 23(3-4), pp.140-146.

Machida, M., and Gomi, K., Eds., 2010. *Aspergillus: Molecular Biology and Genomics*. United Kingdom: Horizon Scientific Press.

Martins, M.L., Martins, H.M., and Bernardo, F., 2001. Aflatoxins in spices marketed in Portugal. *Food Additives and Contaminants*, 18(4), pp.315-319.

Nair, S.C., Bhagobaty, R.K., Nampoothiri, K., Kalaigandhi, V., and Menon, K.R.K., 2014. Detection of Aflatoxin production by fungi in spice samples using HPLC and direct visual cultural methods. *Innovative Romanian Food Biotechnology*, 14, pp.1-12.

Raja, M., Praveena, G., and William, S.J., 2017. Isolation and identification of fungi from soil in Loyola College campus, Chennai, India. *International Journal of Current Microbiology and Applied Sciences*, 6(2), pp.1789-1795.

Ramírez-Camejo, L.A., Zuluaga-Montero, A., Lázaro-Escudero, M., Hernández-Kendall, V., and Bayman, P., 2012. Phylogeography of the cosmopolitan fungus *Aspergillus flavus*: Is everything everywhere? *Fungal Biology*, 116(3), pp.452-463.

Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R., Lima, N., and Venâncio, A., 2007. Identification and characterization of *Aspergillus flavus* and aflatoxins. In: *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*. Formatex, Mumbai.

Rohilla, S.K., and Salar, R.K., 2012. Isolation and characterization of various fungal strains from agricultural soil contaminated with pesticides. *Research Journal of Recent Sciences ISSN*, 2277, p.2502.

Scherm, B., Palomba, M., Serra, D., Marcello, A., and Migheli, Q., 2005. Detection of transcripts of the aflatoxin genes aflD, aflO, and aflP by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *International Journal of Food Microbiology*, 98(2), pp.201-210.

Soni, S.K., 2007. *Microbes: A Source of Energy for the 21st Century*. New India Publishing, New Delhi.

Temu, G.E., 2016. Molecular Identification of *Aspergillus* strains and quick detection of aflatoxin from selected common spices in Tanzania. *Journal of Scientific Research and Reports*, 10, pp.1-8.

Yazdani, D., Zainal, A.M., Tan, Y.H., and Kamaruzaman, S., 2010. Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. *African Journal of Biotechnology*, 9(45), pp.7654-7659.

Yin, Y.N., Yan, L.Y., Jiang, J.H., and Ma, Z.H., 2008. Biological control of aflatoxin contamination of crops. *Journal of Zhejiang University Science B*, 9(10), pp.787-792.