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Analysis of impact of anaerobic condition on the aflatoxin production in *Aspergillus parasiticus* Speare

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ABSTRACT

Aflatoxins are the natural carcinogens that are the best characterized as fungal secondary metabolites. The producers that are responsible for aflatoxin biosynthesis are strongly associated in toxic contamination of essential agricultural products. *Aspergillus parasiticus* is an exclusive fungus that participates in causing hepatic problems in humans and cattle. These mycotoxins are greatly influenced by abiotic stresses. The fungal growth, proliferation and its toxigenicity are highly influenced by these stresses. Present study aimed to restrict the mycelial growth and to prevent aflatoxin preparation in *A. parasiticus* under the anoxic stress. The monosporic strains of *A. parasiticus* were grown in two different Erlenmeyer conical flasks containing Czapek Dox Broth and Czapek Dox Agar under both aerobic and anaerobic conditions. The anoxic condition was maintained using Anaero Bag System. Aflatoxin was isolated after 10 days, and quantitative estimation was done by using High Performance Liquid Chromatography (HPLC). The experimental outcome showed that there was a drastic decrease in both the morphological growth and the aflatoxin biosynthesis of *A. parasiticus* in anoxic state.

Key words: Aflatoxin, Anaerobic, Anaero Bag System, *Aspergillus*, HPLC.

Aflatoxins are a group of naturally occurring carcinogenic fungal polyketide-derived secondary metabolites that frequently contaminate agricultural commodities in tropical and temperate regions of the World (Wang *et al*. 2013; Kumar *et al*. 2018a). The actual occurrence and production of aflatoxins are greatly influenced by environmental factors including weather, geographical locations, substrate composition and agronomic practices (Ross *et al*., 1992; Pande and Mishra, 2018). They are the low molecular weight organic compounds produced by imperfect *Aspergillus* sp and are belong to the furocoumarin cyclopentenone series (Kumar *et al.*, 2017a). Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1) and Aflatoxin G2 (AFG2) are the popular known mycotoxins commonly produced by aflatoxigenic fungi. *Aspergillus flavus* and *Aspergillus parasiticus* are predominantly known for aflatoxin production (Singh *et al*. 2017). *A. nomius*, *A. pseudotamarii*, *A. pseudocaelatus*, *A. pseudonomius*, and *A. bombycis* are some other known aflatoxigenic fungi (Frisvad *et al*. 2019).

Morphologically, *Aspergillus* species are well differentiated (Kumar *et al.* 2017b; Kumar *et al*. 2018b). Periodic consumption of aflatoxin contaminated foods may lead to hepatocellular carcinoma in humans and other animals (Webster and Weber, 2007; Kumar *et al.* 2017c; Kumar *et al*. 2018c). Generally, fungi are aerobic heterotrophs but some are facultative anaerobes. Ecologically also they are well adapted in both aerobic and anaerobic condition. As far as *A. parasiticus* is concern, it being well adapted to grow fast under aerobic condition and its growth under anaerobic condition (low oxygen tension) is underestimated. In this present study, we evaluate the physiological and morphological effects on *A. parasiticus* along with reduction in aflatoxin production.

Monosporic strains of *A. parasiticus* was obtained from Pondicherry University, India. It was grown in two different 250 ml Erlenmeyer conical flasks, each containing 50 ml of autoclaved Czapek Dox Broth (CDB) (TM Media, India). Both broth media were allowed to cool at 45 °C and 500 µl streptomycin (an antibiotic) solutions was used in both flasks to restrict any bacterial growth. The fungi was also inoculated on sterilized Czapek Dox Agar (CDA) media (TM Media, India), for its microscopic analysis under both aerobic and anaerobic conditions (The 15 ml of CDA medium in different sterilized Petri-plates were taken under aseptic region (Fig 1a & Fig 1b). The CDB and CDA media were incubated likewise and in same time period. The one group was incubated in aerobic condition while the other group was incubated under anaerobic condition, at 28 °C for 10 days (Kumar *et al*. 2017b). Anaerobic condition was created

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by using 'Anaero Bag System' (Hi-Media) and 'Anaero Indicator Tablets' (Hi-Media) were used to detect the anaerobic environment. This tablet is an oxygen indicator which remains a pink pill under anoxic condition and changes to purplish-blue in the presence of oxygen (Yoshikawa *et al.* 1982).

After providing suitable incubation period, the percent colony growth inhibition of the *A. parasiticus* was measured in diameter (in cm) on the medium of each Petri plate was calculated by following method (Kumar *et al*., 2017b):

Where $$\text{Percent colony growth inhibition} = \frac{a-b}{b}$$

a =diameter of fungal colony (mean) in control
b= diameter of fungal colony (mean) with plant extract
Percentage of inhibition mycelium dry mass was calculated by the following formula (Kumar *et al*. 2017b):

$$\text{Perccentage of inhibition of growth of test fungi} = \frac{DC-DT}{DC} \times 100$$

Where,

DC = Average increase in mycelial dry mass in the control sample.
DT = Average increase in mycelial growth of the test sample.

After 10 days, aflatoxins were extracted from both media. High Performance Liquid Chromatography (HPLC) was performed to determine their content. Ten grams of *A. parasiticus* culture were blended with the solution containing 50ml methanol (55%) and 20 ml petroleum ether for 10 minutes continuously. The blended mixtures were kept undisturbed for 30 minute and then, the extract filtered by using Whatman filter paper no 1 to remove all mycelia and cell debris. In new falcon tubes, 25 ml aqueous methanol phase and 25 ml chloroform were added, and mixed properly by gently shaking for 2 minutes (Kumar *et al*. 2017b). The falcon tubes were left to separate for next 5 minutes, and aflatoxins were concentrated for HPLC detection. Elution of the matrix loaded in the column was carried out with chloroform: methanol (11.76:0.24) at the rate of 5 ml/min. 5ml of fraction was collected from both aerobic and anaerobic samples and monitored for the absorbance of aflatoxins at 360 nm in UV spectrophotometer, and followed by HPLC for further confirmation. The C18 Polaris column (Ace, 250 mm x 4.6 mm, 5ìm) was used for stationary phase. The samples were run with an isocratic mobile phase consisting of deionized water: methanol: acetonitrile (70:20:10) at a flow rate of 1 ml/min (Rajarajan, *et al.*, 2013). The absorption of samples in HPLC was detected using florescence detector with excitation at 360 nm and emission at 450 nm.

Many extrinsic factors are adversely affecting the growth and proliferation any fungi. Abiotic stress among them is one of potential factors which highly influence the growth and any toxigenic activities. In this approach significant inhibitory effects have been seen in terms of mycelial proliferation which was microscopically investigated and aflatoxin production in *A. parasiticus*. The cultured fungal species was comparatively analyzed under both aerobic and anaerobic conditions. Morphologically, the conidial growth and mycelial growth were highly restricted (Fig1a &1b). These reductions were confirmed by measuring the dry cell weight. As far as colonial growth is concern considerable percentage deterioration has been seen whereas drastic percentage inhibition was also observed. On 6th day of the fungal growth in anaerobic condition the percentage inhibition of mycelial dry weight increased very high with respect to 2nd and 4th day of inhibition. Same in the case, the percentage inhibition of colonial average growth was found high at 6th days (Table1).

The results showed that both the aflatoxin biosynthesis and morphological growth of *A. parasiticus* were significantly affected by anaerobic condition. There were many differences in morphological features in the culture under anaerobic condition in comparison to aerobic condition. The anaerobic condition affects the might affect the growth and propagation of fungi at cellular level. Many



Fig 1 (a): *A. parasiticus* culture (aerobic condition).

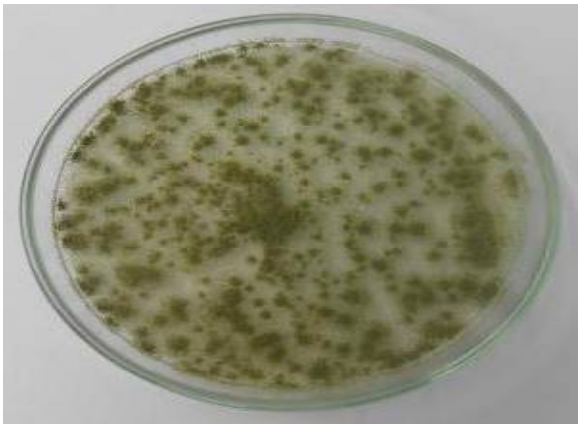


Fig 1 (b): *A. parasiticus* culture (anaerobic condition).

Table 1: Percentage Inhibition in colony growth and mycelial dry weight

Growth→	Colony average growth		Mycelial dry weight	
Days↓	(cm)	Growth Inhibition (%)	(gm)	Inhibitory effect (%)
Control*	6.9	0.00	0.434	0.00
2nd	6.3	8.69	0.413	4.84
4th	5.7	17.39	0.373	14.05
6th	3.4	50.72	0.202	53.46
8th	2.2	68.12	0.183	57.83
10th	1.4	79.71	0.137	68.43

*The fungal cultures were grown under aerobic condition for 10 days

Table 2: Quantitative comparison of amount aflatoxins (AFB1, AFB2, AFG1 and AFG2) produced by *A. parasiticus* in between aerobic and anaerobic condition after 10 days.

Quantitative analysis of aflatoxin		
Aflatoxins	**Aerobic Condition (ng/ml)**	**Anaerobic Condition (ng/ml)**
AFB1	6.39	2.83
AFB2	6.17	0.52
AFG1	2.86	0.28
AFG3	2.54	0.37

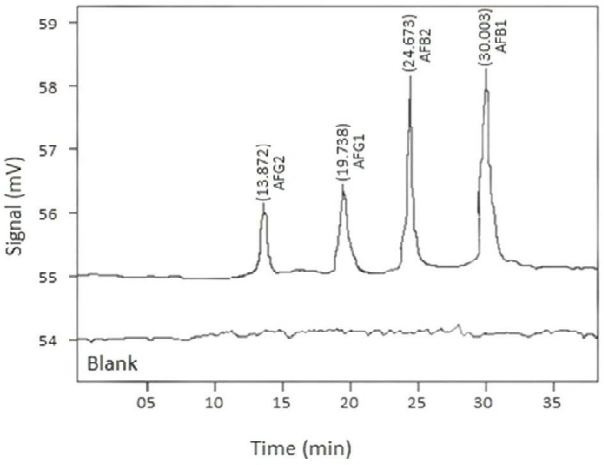


Fig 2 (a): HPLC of different aflatoxins isolated from *A. parasiticus* growth culture in aerobic condition.

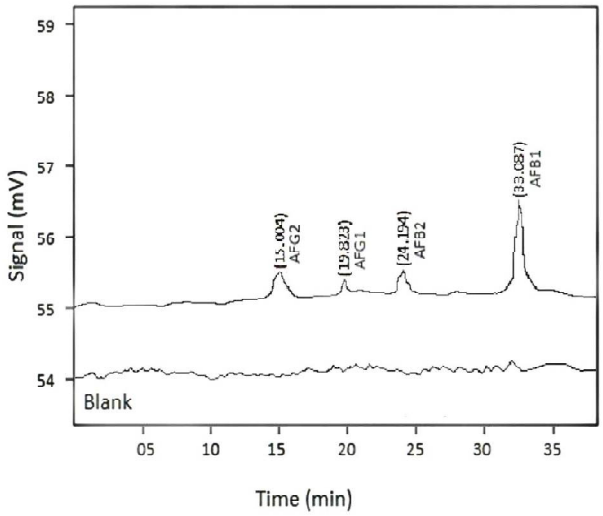


Fig 2(b): HPLC of different aflatoxins isolated from *A. parasiticus* growth culture in anaerobic condition.

biological functions also impede during metabolic activities (Choudhury *et al*. 2018). In some cases, the anaerobic status might also affect the division of mitochondria and biosynthesis of cytochromes necessary for the aerobic growth (Youssef *et al.* 2013) which need to be exploring further. The use of HPLC also enhanced the separation performance and identify the aflatoxins was based on the retention time (RT) in minutes (mins) of the peak areas. All the four aflatoxins i.e. AFB1, AFB2, AFG1 and AFG2 were well separated as shown in Fig 2(a). Under the anaerobic stress condition, considerable reduction in aflatoxins has been shown as compared to aerobic condition. (Fig 2b). The quantitative analysis also confirmed the huge reduction in aflatoxins amount (Table 2). To identify accuracy under anaerobic condition the AFB1, AFB2, AFG1 and AFG2 have shown huge reduction with 2.83, 0.52, 0.28 and 0.37 ng/ml respectively.

The result in this analysis showed the comparative study of *A. parasiticus* under aerobic and anaerobic condition. The percentage degradation of aflatoxins is comparatively more in anaerobic condition. It was observed that the size of mycelium, and amount of conidia formation diminished along with the shape of chonidiospores in anaerobic condition. Inhibition of aflatoxin biosynthesis was further confirmed by HPLC. Significant cutback in amount of aflatoxins AFB1, AFB2, AFG1 and AFG2 have seen. Still further research would require at molecular level.

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