

Full Length Research Paper

Optimization of nutritional and physiological conditions for toxigenic strains of *Aspergillus flavus*

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The objective of the present work was to evaluate the capacity of two isolates of *Aspergillus flavus* from sugar cane (SC), SC-3 and SC-5 to produce aflatoxin under different culture conditions. Aflatoxin B₁ (AFB₁) production was determined in four different media and culture conditions (temperature and pH) were varied to achieve maximal toxin production. AFB₁ was extracted directly from the cultures with chloroform. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used to identify AFB₁. It was observed that *A. flavus* cultures SC-3 and SC-5 produced highest AFB₁ when grown in potato dextrose medium (PD) and maximum mycelium production in sabouroud dextrose medium (SD). Supplementation of potato dextrose medium with peptone (PD+P) and sodium nitrate (PD+SN), AFB₁ production increased by 27 and 20% respectively when compared to the control. The effect of pH and temperature on aflatoxin production by *A. flavus* was examined in peptone containing PD medium. The results concluded that AFB₁ production and mycelial growth was maximum at pH 5.6 when incubated at 27°C in potato dextrose medium supplemented with peptone.

Key words: Aflatoxin B₁, *Aspergillus flavus*, mycotoxins.

INTRODUCTION

Mycotoxins are the secondary metabolites of fungi, which are highly toxic and widely distributed in food and feed. Among the reported mycotoxins, Aflatoxins are mutagenic and carcinogenic to animals and humans (Jelinek et al., 1989). They are produced by the toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*, the well-known contaminants found in agricultural commodities. Aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂) are the four major naturally occurring aflatoxins among 28 different types of aflatoxin identified and characterized (Yiannikouris and Jouany, 2002; Basappa, 2009). All *Aspergillus* species are not toxigenic in nature and

various species of *Aspergillus* produces different toxins at varying concentration (Bragulat et al., 2001; Medina et al., 2006).

Ingestion of aflatoxin contaminated food leads to hepatocarcinogenic, mutagenic, teratogenic and genotoxic effects in animal system. Aflatoxin in human causes acute aflatoxicosis which involves failure of liver and gastrointestinal complications (Yiannikouris and Jouany, 2002). Aflatoxin B₁ has been defined as potent carcinogen by the International agency for research on cancer (IARC) and is the most deleterious among other Aflatoxins (Payne, 1992, 1998; Widstrom, 1996).

Park and Bullermann (1983) reported that substrate on which the fungi colonizes holds the prime importance in the toxin production. Several other factors such as temperature, water availability, nutrients and pH also influence the fungal efficacy in toxigenesis. Mechanical damage and insect invasion favors the colonization of the

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toxigenic fungi on to the substrate.

Optimal conditions of nutrition and physiological parameter varies for different strains (Chinnasamy et al., 2011). This makes it necessary to optimize the conditions for the newly isolated toxigenic strains SC-3 and SC-5. Considering the importance of evaluating alternative and efficient methods for the characterization of toxigenic potential, the objectives of this study were to evaluate the growth capacity and toxigenic potential of two *A. flavus* strains isolated from sugarcane in different culture conditions.

MATERIALS AND METHODS

Fungi isolation and identification

The fungi used in this study were isolated from the Sugarcane samples collected from retail shop located in Vellore region (Tamilnadu, India) and these strains SC-3 and SC-5 were independently confirmed as *A. flavus* by the Agharkar Research Institute Pune, India. Cultures were maintained on Potato dextrose agar (PDA) medium slants at 4°C.

Culture media

The following culture media were used in the present work: Czapek dox broth (CD): 3 g sucrose, 0.2 g sodium nitrate, 0.1 g dipotassium phosphate, 0.05 g magnesium sulphate, 0.05 g potassium chloride, and 100 ml distilled water. Sabouraud dextrose broth (SD): 1 g peptone, 4 g dextrose, and 100 ml distilled water. Malt extract broth (MD): 0.6 g malt extract, 0.18 g maltose, 0.6 g dextrose, 0.12 g yeast extract, and 100 ml distilled water. Potato dextrose broth (PD): 4 g potato starch, 2 g dextrose, and 100 ml distilled water. The media were autoclaved at 121°C for 15 min.

Culture conditions

A loop full of *A. flavus* Spores SC-3, SC-5 was taken and diluted to 10^{-4} with 10 ml of water, from which 100 µl was withdrawn and inoculated on the potato dextrose agar plate by spread plate method. Plates were incubated at 27°C and colonies were formed after 24 h of incubation. Mean while 20 ml of four different medium czapek dextrose, sabouraud dextrose, malt extract, and potato dextrose broth were prepared. Using a cork borer of 5 cm in diameter the separated colonies from the Petri plate was cut and inoculated into four different medium and incubated at 27°C for 5 days, each experiment was carried out in triplicates.

Since Potato dextrose broth was found to be suitable for the toxin production, further studies was carried out to determine whether natural peptone or chemical nitrogen ingredients were responsible for enhanced mycelia growth. Potato dextrose medium was supplemented with 10 g/l peptone (PD+P) and 2 g/l sodium nitrate (PD+SN) in the same way as mentioned above. The growth of *A. flavus* and AFB₁ production were standardized on Potato dextrose supplemented with peptone at various pH and temperature. Potato dextrose supplemented with peptone was inoculated with a single spore suspension and incubated at the required temperature (20, 27 and 37°C) and also with different pH level (4, 5 and 5.6) for 5 days. After the incubation period, mycelium was harvested from the medium using Whatmann no.1 paper and the dry weight was calculated by placing it in hot air oven at 60°C. Decrease in weight

was measured for every one hour, until a constant dry weight was attained.

Chromatographic analysis of aflatoxin B₁

Thin layer chromatography

After the incubation period the culture supernatant and the mycelium separated using Whatman no.1 paper. To the supernatant twice the volume of chloroform was added and extracted. The extract was concentrated to the final volume of 5 ml and the amount of AFB₁ in the samples was analyzed using UV spectrophotometer at 360 nm. Qualitative analysis of AFB₁ using thin layer chromatography (TLC) was performed along with a standard AFB₁ (Supelco, Bellefonte, PA, USA). Ten microliter of the extract was applied on activated TLC plate (Silica gel 60 F254, Macherey-Nagel, Germany) and chloroform: Acetone (85:15) was used as a solvent system. After developing the chromatogram, the plates were viewed under U.V light (360 nm) to detect the characteristic fluorescence of aflatoxin B₁ (Lin and Dianese, 1976). After purification with silica gel column, 75% AFB₁ was recovered.

High performance liquid chromatography

The aflatoxin was concentrated and loaded on the matrix, elution was carried out with chloroform: methanol (11.76:0.24), at a flow rate of 5 ml/min. A total of 25 fractions were collected (5 ml each) and monitored by taking the absorbance at 360 nm in UV spectrophotometer. The purity of AFB₁ in the fraction was confirmed by High performance liquid chromatography (HPLC, Waters 1525) with UV detector at 360 nm as per the instructions given in Supelco instruction manual. The stationary phase was C18 Polaris column. A sample of 20 µl was injected, deionized water: acetonitrile: methanol (60:20:20) was used as mobile phase at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Isolation and identification of *A. flavus* and AFB₁ production

The toxigenic strains SC-3 and SC-5 used in the present study were isolated from sugarcane sample and identified as *A. flavus*. On the basis of the colony appearance, morphological characters and conidial arrangements showed that the isolates were *A. flavus*, which was confirmed by the Agharkar Research institute, Pune, India (Figure 1). The isolates were investigated for the production of AFB₁ by growing in YES broth (2% yeast extract, 15% sucrose) in 250 mL conical flask, incubated at room temperature for 5 days. The chloroform extract showed the presence of AFB₁ spot on TLC at R_f value 0.7 (Figure 2) The presence of AFB₁ was also confirmed by comparing with standard AFB₁ on TLC as well as HPLC along with the sample (The isolated *A. flavus* was found to produce AFB₁ at a concentration of 5 µg/ml in YES medium after 7 days of incubation). After purification with silica gel column, 75% AFB₁ was recovered. The purified AFB₁ gave a single peak in HPLC at retention

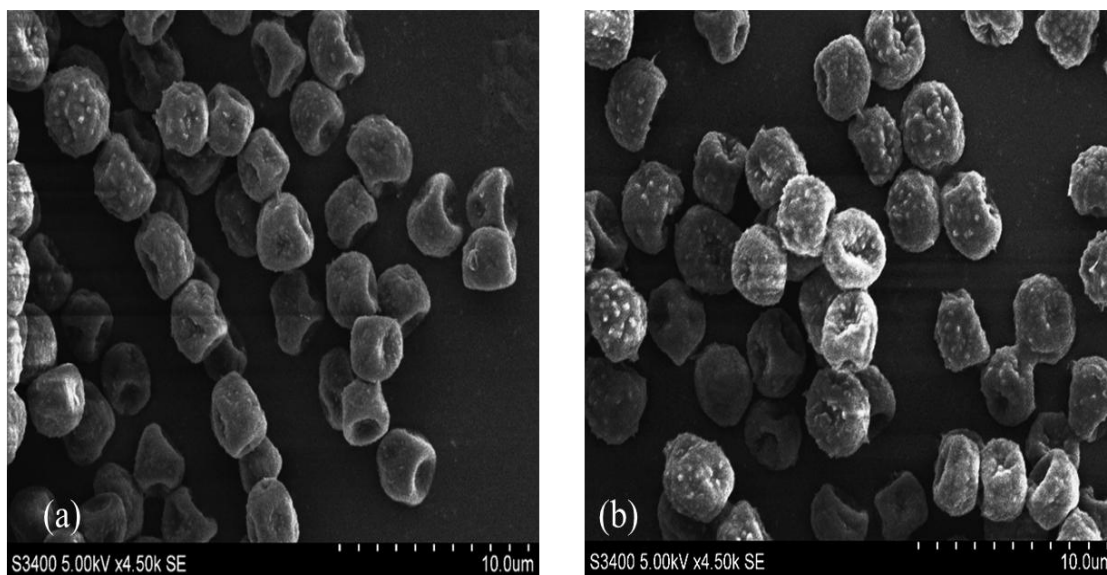


Figure 1. Scanning electron microscope images of fungal isolates (a) SC-3 and (b) SC-5.



Figure 2. Chromatography of AFB₁ spot in the presence of chloroform extract. Lane 1, Standard AFB₁, lane 2, AFB₁ sample extracted from *A. Flavus*. TLC was run in the solvent system of chloroform:acetone (85:15).

The effects of culture media on aflatoxin B₁ production and mycelium dry weight estimation

The effect of the culture media on growth and AFB₁ production was studied by inoculating *A. flavus* strain SC-3 and SC-5 into 20 ml of each czapek Dox, sabouraud dextrose, malt extract, potato dextrose broth and incubated at 37°C for 5 days. Results are presented in Table 1. After the filtration and separation of mycelium from the supernatant the sabouraud dextrose produced maximum mycelium when compared with the other medium. While potato dextrose produced the maximum AFB₁ in the medium. According to the results of Joffe and Lisker (1969) greater aflatoxin accumulation was observed in Czapek liquid medium at an initial pH of 4.0. Similarly, Reddy et al. (1971) reported that pH 4.5 produced higher aflatoxin in synthetic low salt (SL) medium. A number of experts has reported on factors affecting aflatoxin production on synthetic media (SH) and they proved SH medium is apparently unfavourable for high aflatoxin yield (Adye and Mateles, 1964; Ciegler et al., 1966; De longh et al., 1962). Whereas aflatoxin production is high in crude media than synthetic media (Davis et al., 1966) this is because synthetic media mainly contains precipitated salts, which are unfavorable for high yields of aflatoxin (Lee et al., 1966; Tulpule, 1969). To determine whether the natural peptone or chemical nitrogenous source responsible for enhancing the mycelium and toxin production, we supplemented the potato dextrose medium with peptone (PD+P) or sodium nitrate (PD+SN). Mycelial growth and toxin production after 5 days incubation at 27°C was observed maximum

time of 8.67 min (Figure 3).

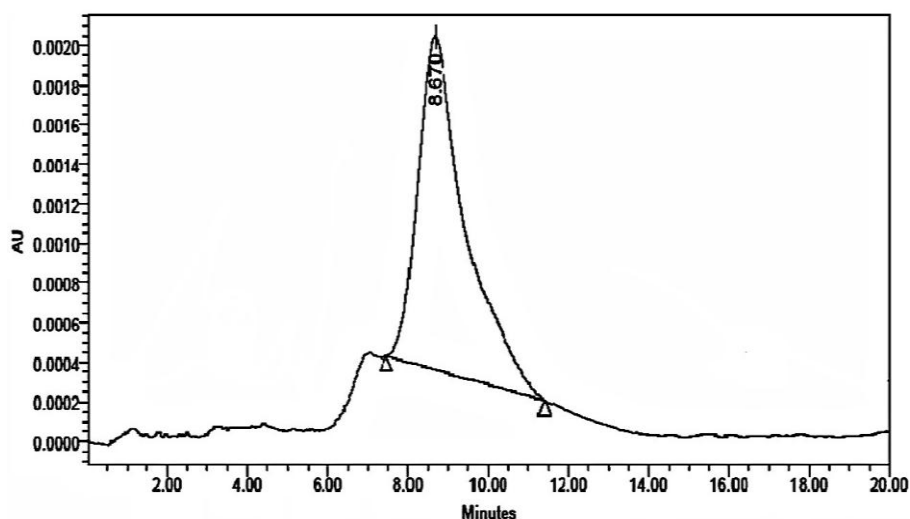


Figure 3. High pressure liquid chromatography of AFB₁ isolated from sugarcane sample. AFB₁ peak obtained at a retention time of 8.67.

Table 1. Dry weight estimation and Aflatoxin production by *Aspergillus flavus* SC-3 and SC-5 on different growth medium.

<i>A. flavus</i>	Dry weight of mycelium (g)				AFB1 Concentration (µg/ml)				
	C.D	S.D	M.D	P.D	C.D	S.D	M.D	P.D	YES
SC-3	0.09±0.014	0.14±0.007	0.067±0.008	0.126±0.024	0	2.55±0.225	2.1±0.23	4.2±0.38	4.9±0.2
SC-5	0.12±0.016	0.12±0.009	0.052±0.005	0.108±0.01	0	2.65±0.176	2.1±0.125	4.05±0.36	(nd)

*CD – Czapek dox broth, SD- Saubaraud dextrose broth, MD- Malt extract broth, PD- Potato dextrose broth, YES – Yeast extract sucrose broth, (nd) - not detected.

Table 2. Effect of the nitrogen source supplement on growth and AFB₁ production by *Aspergillus flavus* SC-3 and SC-5.

<i>A. flavus</i>	Dry weight of mycelium (g)			AFB1 Concentration (µg/ml)		
	PD	PD+P	PD+S	PD	PD+P	PD+SN
SC- 3	0.078±0.024	0.134±0.009	0.115±0.002	4.2±0.36	5.75±0.42	4.75±0.25
SC- 5	0.078±0.015	0.135±0.006	0.112±0.003	4.05±0.36	6.3±0.3	4.35±0.3

*PD- Potato dextrose broth, PD+P-Potato dextrose with peptone, PD+SN- Potato dextrose with sodium nitrate.

in potato dextrose supplemented with peptone (PD+P) exhibiting a significant yield ($P < 0.05$) by T-test, compared to the medium supplemented with sodium nitrate (Table 2). This shows toxigenic potential of *Aspergillus* sp can be well demonstrated in potato dextrose medium composed of organic nitrogen source. According to Reddy et al. (1971) the synthetic low salt (SL) medium which contained low concentration of inorganic salt when compared to synthetic high salt (SH) medium, favored aflatoxin production. This is mainly because of the inorganic salt like dihydrogen potassium phosphate and

magnesium sulphate this reduces the aflatoxin production. In the experiment the inorganic nitrogen source when given additionally to the potato dextrose broth (PD+SN) lead to less aflatoxin production than potato dextrose medium supplemented with peptone (PD+P).

The presence of inorganic nitrogen source may also be the reason for the less aflatoxin production. As a result we concluded that the medium lacking peptone showed significant decrease in the mycelia dry weight and aflatoxin production (Table 2).

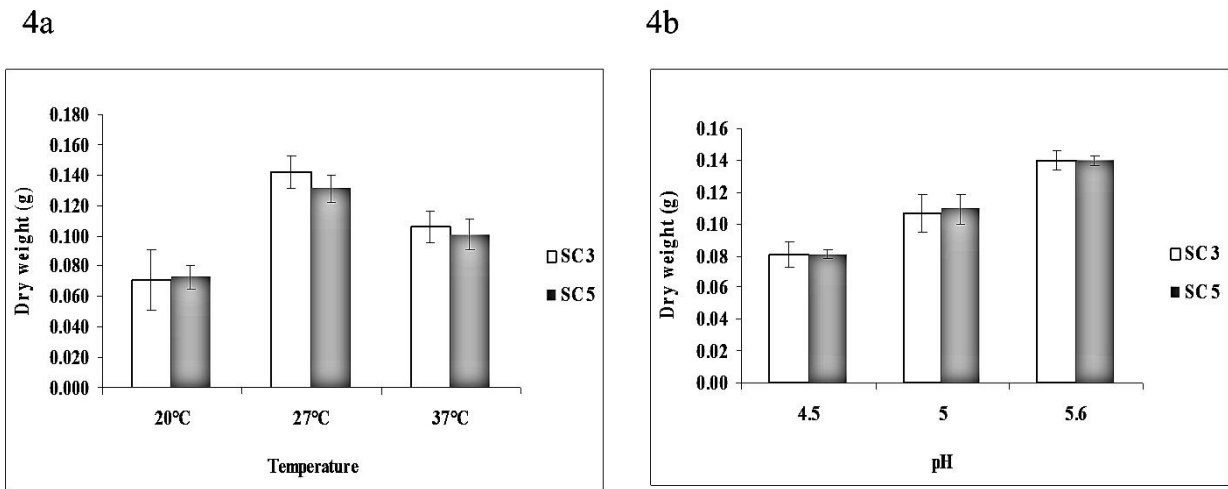


Figure 4. Determination of fungal dry weight based on temperature and pH. A) Incubation of SC-3 and SC-5 in potato dextrose broth with peptone under different temperature condition; B) Incubation of SC-3 and SC-5 in potato dextrose broth with peptone under different pH condition.

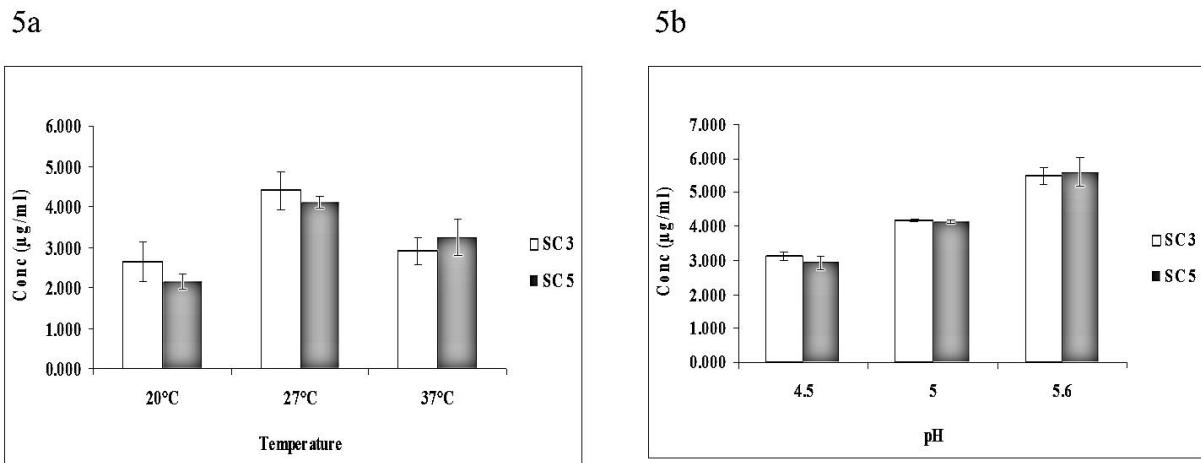


Figure 5. Determination of Aflatoxin based on temperature and pH. A) Incubation of SC-3 and SC-5 in potato dextrose broth with peptone under different temperature condition. B) Incubation of SC-3 and SC-5 in potato dextrose broth with peptone under different pH condition.

Interaction of independent variables (pH and temperature)

The pH and temperature of the medium is another important factor influencing fungal growth and aflatoxin production. The isolates S.C (3) and S.C (5) produced aflatoxin B₁ in the following conditions at 20, 27 and 37°C with a pH of 4.93 and incubation time was 5 days. With different temperature value tested 27°C was found to be appropriate for aflatoxin production (Figures 4a and 5a). The results of Gqaleni et al. (1996), showed 30°C is

optimal temperature for the production of aflatoxin in the CYA and YES media. In a similar way effect of pH was studied by adjusting the pH 4.5, 5, 5.6 on SC-3 and SC-5. The aflatoxin production and mycelium growth on PDA medium was studied. Among the different pH tested, 5 and 5.6 favored aflatoxin production (Figures 4b and 5b); Molina and Giamuzzi (2002) studied the effect of pH (5.5 and 5.9) on the production of aflatoxin B₁, and reported that pH 5.9 gave the highest aflatoxin levels. Lie and Marth (1968) reported that aflatoxin development on a casein substrate was high at extreme acidic or alkaline

pH (pH 2 and 9.5). It has also been reported that aflatoxin yields on a czapek-dox medium increased several-fold when the pH was changed from 7.4 to 4.0 (Joffe and Lisker, 1969). The effect of pH and temperature on growth of mycelium and aflatoxin production in potato dextrose with peptone medium is summarized in Figures 4 and 5. Maximal growth occurred at an initial pH of 5.6. However, the mold grew well over the entire pH range. We concluded that the initial pH level of 5.6 and 27°C favored aflatoxin and mycelium production. In general saprophytic fungi prefer acidic pH, therefore in the present study pH below 7 were used.

Conclusion

A. flavus isolates SC-3 and SC-5 produced aflatoxin B₁ in different conditions and also produced higher concentration of aflatoxin B₁ which was determined by qualitative tests such as thin layer chromatography and high performance liquid chromatography method proved to be very efficient in detecting aflatoxin B₁. The results of the present study suggest the use of the potato dextrose supplemented with peptone medium with pH 5.6, at 27°C for maximal aflatoxin and mycelium production with an incubation time of 5 days to detect the toxigenic potential of *A. flavus*.

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