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ORIGINAL ARTICLE

Production, Purification and Characterization of aflatoxins by *Aspergillus flavus* isolated from Groundnut

Renu Khare[a,b]*, Mukesh Agarwal[b], Sameer S. Bhagayavant[a] Jaggi Lal[c]
*,[a] School of Studies in Biotechnology, Jiwaji University, Gwalior-474 011, Madhya Pradesh, India
[b] Biotechnology Division, Defence Research and Development Establishment, Jhansi Road Gwalior-474 002, Madhya Pradesh, India
[c] School of Studies in Chemistry, Jiwaji University, Gwalior-474022, Madhya Pradesh, India
Email: merenukhare@gmail.com

ABSTRACT
A fungus Aspergillus flavus was isolated from groundnut samples and characterized morphologically using fluorescent microscope and carbohydrate assimilation test. Isolated fungal culture was screened for the production of aflatoxins. Obtained Aflatoxins were characterized by TLC, UV-visible spectroscopy and FT-IR spectral studies. Culture conditions for the production of aflatoxins were optimized using physical parameters such as temperature, pH, carbon sources and nitrogen sources. Aflatoxins were produced 141-252 mg/100 mL as crude aflatoxins in a nutrient solution consisting of 20% sucrose 5% peptone and 2% of yeast extract. The purpose of this report is to describe the effect of different growth and cultural conditions on the production of aflatoxins in submerged culture, for the studies of the chemistry and toxicology of these compounds, therefore it is desirable to have them available in gram quantities.
Keywords *Aspergillus flavus, Mycotoxins, Aflatoxins, Thin Layer Chromatography, Submerged culture*

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INTRODUCTION

Mycotoxins are the secondary metabolites produced by moulds. They exert toxic effects on humans and animals, which is referred to as mycotoxicosis. The severity of manifestation of mycotoxins depends on their toxicity. The presence of the mycotoxins in foods has been an international concern from the date of discovery. In January 1999, the United Nation Special Commission (UNSCOM) on Iraq mentioned weaponization of an aflatoxin in the synoptic report. Among the various mycotoxins identified aflatoxins have assumed a great significance in context to their most devastating effects on animal live stock, poultry and human [1]. After few months, the death of more than 1,00,000 young Turkeys in poultry farms in England, an apparently new disease that was termed as "Turkey X disease" appeared. Speculations made during 1960 regarding the nature of the toxins suggested that it might be of fungal origin. In fact, in 1961 the toxin producing fungal species were identified as *Aspergillus flavus* and the produced toxins were given the name aflatoxins by virtue of its origin (*A. flavus* → Afla) [2].

The aflatoxins are toxic metabolites produced by various strains of *Aspergillus flavus* and related micro organisms. *Aspergillus flavus* is an important *Aspergillus* species having aflatoxin producing capability. Aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 (Figure 1) are a group of toxic, carcinogenic, teratogenic, and mutagenic polypeptide secondary metabolites with health hazards to animals and humans and can adversely affect agricultural productivity [3, 4]. Aflatoxin B_1 was evaluated as a class 1 human carcinogenic activity [5]. The incidence of aflatoxin in food and feed is relatively high in tropical and subtropical regions where the warm and humid climate provides optimal conditions for the growth of moulds [6]. India is an ideal country for problems of aflatoxin to develop since it has high moisture and temperature level during the monsoon season and often inadequate storage facilities.

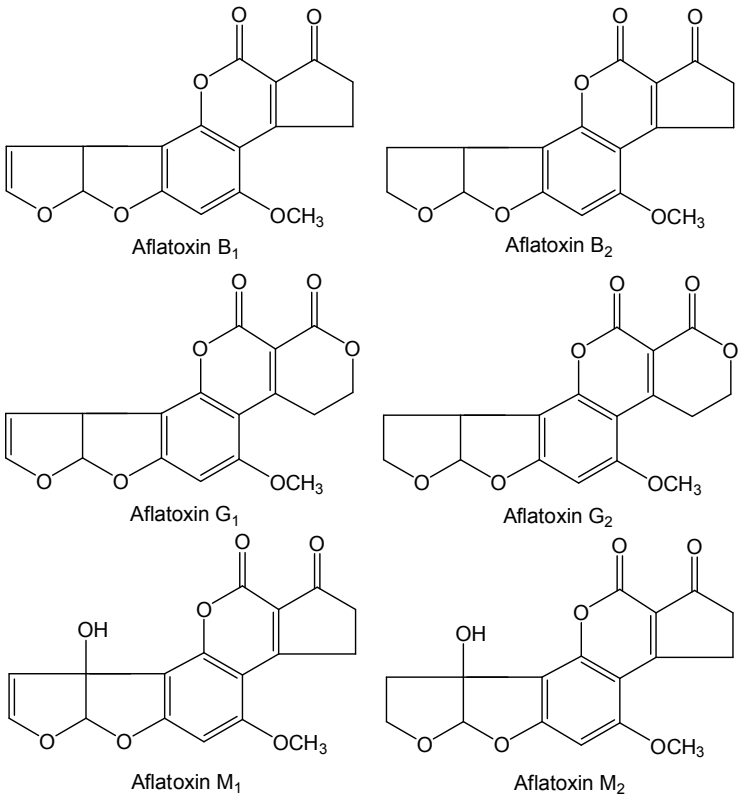


Fig. 1. Chemical structure of various aflatoxins

Chemically, aflatoxins are difuranocoumarin derivatives produced via a polyketide pathway [7, 8]. Recently, coumarin derivatives have been evaluated for the treatment of human immunodeficiency virus [9], due to their ability to inhibit human immunodeficiency virus-1 integrase (HIV-1 IN) in future aflatoxins may be utilized in medical field.

In the present communication groundnut samples were collected and screened for aflatoxin producing microorganisms. The obtained microorganism *A. flavus* was characterized and used in the production of aflatoxins. The aflatoxins were identified by TLC and characterized by UV-visible and FTIR spectroscopy.

MATERIAL AND METHODS

General remarks

Various, sugars, viz. D-glucose, D-xylose, Arabinose, D-mannose, Sucrose, D-galactose, Maltose, D-fructose, D-ribose, Lactose, Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB), yeast extract, peptone, malt extract and standard aflatoxins were purchased from HiMedia chemical company, TLC plates were purchased from Merck, solvents chloroform and acetone were purchased from Rankem. Demineralized water was used throughout the study. Fluorescent microscope (AXIOSCOP 2 MAT) was used for morphological characterization, UV spectroscopy and Fourier Transform Infrared Spectroscopy (Axiovision) for molecular characterization.

Sample collection and preparation

In present study seven aflatoxigenic isolates of *A. flavus* strains was isolated from different food crop samples collected from local market of Gwalior, Madhya Pradesh, India which was detected by PCR. These strains were screened to produce aflatoxins on different media for high yield and maximum aflatoxin production, which was obtained by *A. flavus* II strain in YESP broth. Therefore for *A. flavus* II strain culture, different growth and cultural conditions were optimized.

Erlenmeyer flasks (250 mL) containing 100 mL of medium in each flask were kept and their mouths were plugged with cotton plugs and autoclaved for 15 min at 20 psi. All flasks containing sterile media were incubated with 1 mL of spore suspension from 6-7 days old cultures of *A. flavus* and incubated at 10-12 days at 28°C±2 in stagnant/rotatory shaker. Experiments were carried out in triplicate and results were reported as average of all the values.

A. flavus culture used in the experiment was maintained in proper conditions throughout; the cultures isolated from groundnut samples were maintained on Potato Dextrose Agar media and then stored at 4 °C. Sub-culturing of culture *A. flavus* was performed in every 15 days.

Characterization of fungal culture

Morphological characterization of isolated fungal culture

Isolated fungal culture was characterized morphologically as in figure 2. Under fluorescent microscope (AXIOSCOP 2 MAT) and the results were matched with literature.

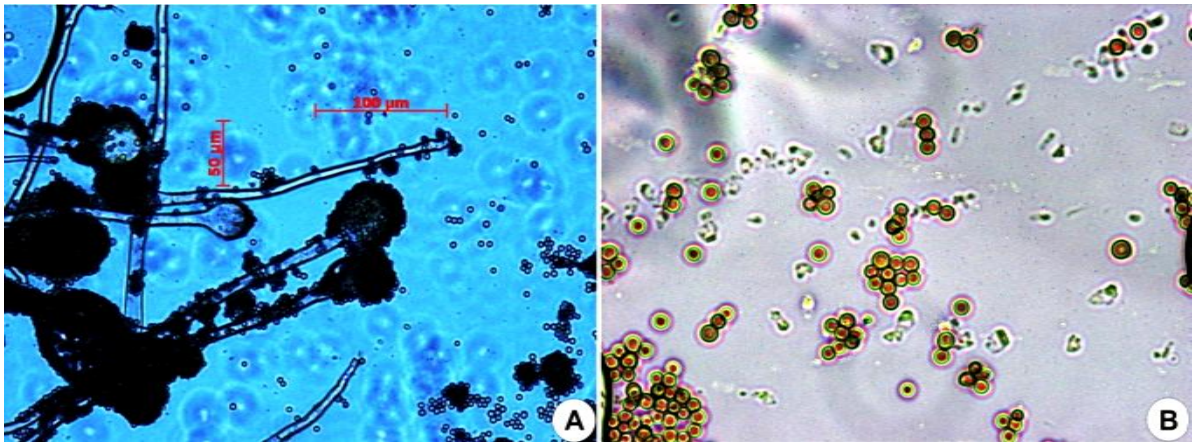


Fig. 2. *Aspergillus flavus* under fluorescent microscope (A) Conidiophores (B) Conidia

Biochemical characterization of isolated *A. flavus*

Carbohydrate assimilation test was used for the biochemical characterization of the isolated fungal culture [10]. The test provides the main apparatus for identification and classification (Figure 3). In mycological work tests are currently being developed for use in the classification of some critical groups especially among the higher fungi. Biochemical tests have been used to classify and identify various groups of fungi. The biochemical characteristic in this study offer possibilities for identification and classification of the fungi.

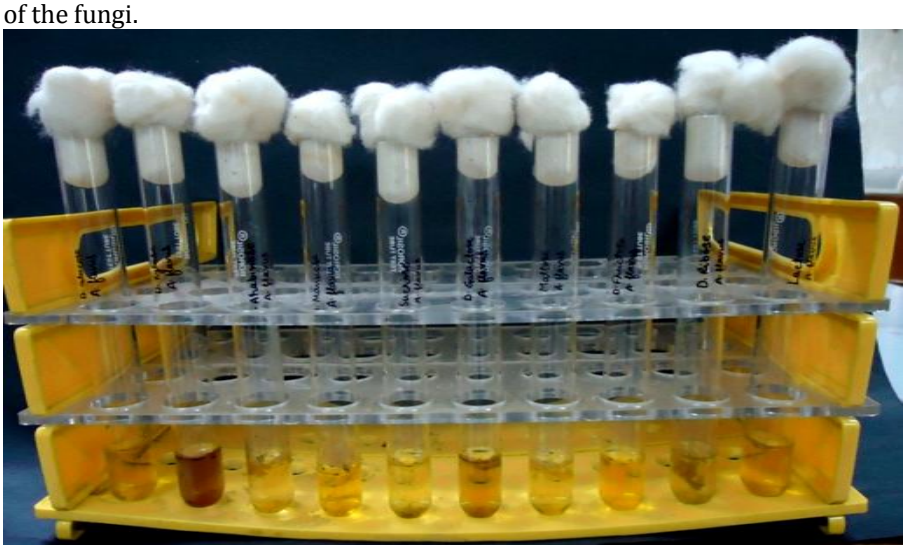


Fig. 3. Biochemical characterization of isolated *A. flavus* from groundnut sample

Vegetative mycelia of the obtained fungal culture were obtained by incubating agar blocks (5×5mm) with mycelia in 2.0 mL of PD broth at 28°C±2 for 24-48 h. The mycelia were washed three times by sterilized distilled water before testing.

In this method the medium consisted of 2.4 mM KCl, 4.2 mM $NH_4H_2PO_4$, 0.8 mM $MgSO_4.5H_2O$, supplemented with 0.01mM $CuSO_4.5H_2O$ and 0.022 mM $ZnSO_4.7H_2O$. Bromo cresol purple dye was added as an indicator at a concentration of 40 mg/L and the medium was autoclaved at 121°C for 10 minutes. Sterilized carbohydrates were added to the medium to a final concentration of 1% (w/v) pH was adjusted to 3.2 using NaOH (0.5 M), HCl (0.5 M) and 2 mL portions of the medium were dispensed into 10 mL test tubes. The tubes were inoculated with fungal mycelia and control tubes for each fungus and carbohydrate were also prepared. All the tubes containing the test mixture were incubated at 20 °C for about 14 days. A change in the color of the medium to orange or yellow was taken as positive results (table 1), a change to pink or purple color was considered a negative results.

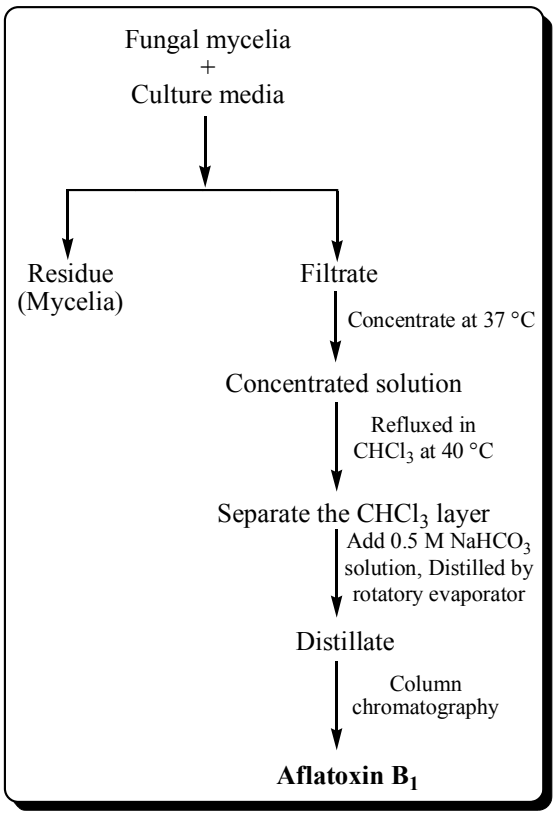
Table 1 Biochemical characterization of isolated fungus (*A. flavus*) using sugars

Entry	Name of sugar	Test
1	D-glucose	-
2	D-xylose	+
3	D-arabinose	-
4	D-mannose	-
5	Sucrose	-
6	D-galactose	-
7	Maltose	-
8	D-fructose	-
9	D-ribose	-
10	Lactose	-

- Negative test *;* + Positive test

Extraction of aflatoxins

After incubation culture was filtered using Whatman No.1 filter paper. Filtrate was concentrated to 37 °C using rotatory evaporator and in the concentrated solution about 20 mL of chloroform was added and refluxed for about 40-60 °C using water condenser for 20-30 min. The mixture was taken in separating funnel and separate chloroform layer containing crude toxins was eluted. The chloroform was recovered using rotatory evaporator. The concentrated culture filtrate was shaken repeatedly with 150 mL volumes of chloroform and the extraction was repeated 2 or 3 times. The chloroform extracts obtained were mixed and filtered through Whatman No. 1 filter paper. From the filtered chloroform extracts, the toxin was extracted by shaking it several times with 0.5 molar sodium bicarbonate solutions. All the lipid materials were removed by filtration after keeping the sodium bicarbonate extract over night in a separating funnel. The extracts was pooled and concentrated, thus the crude toxin was isolated. Purification of crude toxin was done by column chromatography it includes column preparation, capturing of the aflatoxins and washing was done following the Aflaprep® (R-Biopharm) procedure purified aflatoxins (Scheme 1).
Aflatoxin detection done by Thin layer chromatography (TLC).Thus TLC plates obtained were examined and compared with standard samples of aflatoxins. As reported in literature, the presence of aflatoxins may be detected by observing the TLC plate under strong UV illumination and it fluorescence as bluish green spots [11].



Scheme 1. Extraction of aflatoxins from *A. flavus* species

RESULTS

Optimization of culture conditions for the growth of *A. flavus* (II) and aflatoxin production

The optimization for aflatoxin production was performed based on the modification of the physical parameters supplementation of nutrients. The effect of physical factors was determined by modification of temperature, pH of the media effect of different carbon source and nitrogen source on aflatoxin production & biomass were also determined in basal media of Yeast extract. Here the Carbon source was sucrose and nitrogen source was peptone at different concentration.

Effect of different media on aflatoxin production by isolated culture of *A. flavus* (II)

Aspergillus flavus for aflatoxin was submerged in four different media Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB), Yeast Extract Sucrose Peptone Broth (YESPB) and Malt Extract Broth (MEB). The medium in 250 mL shake flask was sterilized at 15 lbs for 15 min. The flask was cooled and then inoculated with 1 mL spore suspension of *A. flavus* (II) and incubated at 28±2 °C in rotatory shaker for 10-12 (Table 2).

Table 2 Effect of different media on aflatoxin production by isolated[a] *A. flavus* (II) strain

Entry	Media	[b] Stationary culture		[c]Shake cuture	
		[d]Mycelial dry wt.	[e]Crude aflatoxins	[d]Mycelial dry wt.	[e]Crude aflatoxins
1	[f]PDB	0.35	0.075	0.32	0.071
2	[g]SDB	0.82	0.132	1.42	0.121
3	[h]MEB	1.32	0.224	0.32	0.122
4	[i]YESPB	1.48	0.252	0.75	0.210

[a] Isolated from groundnut samples.
[b] In incubator.
[c] In rotatory shaker at 150rpm
[d] Weight of dry mycelia was represented in g/100 mL.
[e] Weight of crude aflatoxin was reported in g/100 mL.
[f] PDB - Potato Dextrose Broth.
[g] SDB - Sabouraud Dextrose Broth.
[h] MEB - Malt Extract Broth.
[i] YESPB - Yeast Extract Sabouraud Peptone Broth.

Effect of temperature on aflatoxin production by A. flavus (II) strain in YESPB media

In order to study the effect of temperature on aflatoxins production and fungal growth experiment was carried out at 5 °C intervals in the range of 20 to 50 °C. The flasks were incubated in an incubator for stationary culture and in a rotatory shaker for shake culture at 150 rpm. The maximum aflatoxins production observed at 30 °C in a stationary culture (Table 3).

Table 3 Effect of temperature on aflatoxin production by *A. flavus* (II) in [a]YESPB media.

Entry	Temperature (^{O} C)	[b]Stationary culture		[c]Shake culture	
		[d]Mycelial dry wt.	[e]Crude flatoxins	[d]Mycelial dry wt.	[e]Crude aflatoxins
1	20	0.14	0.114	0.15	0.104
2	25	0.28	0.230	0.17	0.180
3	30	1.36	0.246	0.31	0.214
4	35	1.48	0.176	1.32	0.152
5	40	1.54	0.172	1.54	0.143
6	45	1.64	0.189	1.75	0.154
7	50	1.76	0.186	1.80	0.164

[a] YESPB - Yeast Extract Sabouraud Peptone Broth.
[b] In incubator.
[c] In rotating shaker at 150rpm
[d] Weight of dry mycelia was represented in g/100 mL.
[e] Weight of crude aflatoxins was reported in g/100 mL.

Effect of pH on aflatoxin production by *A. flavus* (II) strain in YESPB media

For the growth of fungi and aflatoxins production pH is an important factor. Fungi must be capable of producing sufficient biomass at optimum pH. To determine optimum pH, *A*spergillus was cultivated in a

250 mL flask containing 100 mL medium with different pH ranges from 3.0-7.0. The pH of the medium was adjusted using 1N HCl or 1N NaOH. The flasks were incubated at 28±2 °C in rotatory shaker for 2-3 weeks at 150 rpm. At the end of incubation period, the content of each flask was filtered, mycelia weight was determined and cell filtrate is used for aflatoxin extraction. The initial pH of the medium did not influence significantly either toxin production or growth. Maximal growth was observed at pH 5 and 6 (Table 4).

Table 4 Effect of different pH on aflatoxin production by *A. flavus* (II) strain in [a]YESPB media

Entry	Initial pH	Final pH	[b]Stationary culture		[c]Shake culture	
			[d]Mycelial dry wt.	[e]Crude aflatoxins	[d]Mycelial dry wt.	[e]Crude aflatoxins
1	5.0	3.0	0.00	0.000	0.00	0.000
2	5.0	4.0	0.68	0.119	0.66	0.010
3	5.0	5.0	0.76	0.242	0.82	0.101
4	5.0	6.0	0.92	0.250	0.96	0.133
5	5.0	7.0	0.89	0.212	0.92	0.124

[a] YESPB - Yeast Extract Sabouraud Peptone Broth.
[b] In incubator.
[c] In rotatory shaker at 150rpm
[d] Weight of dry mycelia was represented in g/100 mL.
[e] Weight of crude aflatoxins was reported in g/100 mL.

Effect of sucrose concentration on aflatoxin production by *A. flavus* (II) strain in YESPB media
In the present work sucrose was selected as carbon source to study the effect of growth of fungal cultures and aflatoxin production. For this experiment sucrose was supplemented at various concentrations 10, 15, 20, 25 and without sucrose in the media (Table 5). From the table it is clear that maximum growth aflatoxin production was observed in 25 %.

Table 5 Effect of sucrose concentration on aflatoxin production by *A. flavus*

Entry	Percentage of sucrose	[a] Stationary culture		[b] Shake culture	
		[c]Mycelial dry wt.	[d]Crude aflatoxins	[c]Mycelial dry wt.	[d]Crude aflatoxins
1	0	0.04	0.114	0.04	0.104
2	5	0.36	0.162	0.21	0.122
3	10	1.42	0.171	1.32	0.141
4	15	1.54	0.173	1.44	0.143
5	20	1.49	0.254	1.49	0.226
6	25	1.52	0.256	1.52	0.234

[a] In incubator.
[b] In rotatory shaker.
[c] Weight of dry mycelia was represented in g/100 mL.
[d] Weight of crude aflatoxins was reported in g/100 mL.

Effect of peptone concentration on aflatoxin production by *A. flavus* (II) strain in YESPB media
In the above experiment peptone was used as nitrogen source in supplementation for fungus. In order to test the best suitable concentration of peptone growth of the fungal culture was maintained at concentrations 1%, 2%, 3%, 4%, 5% and without peptone (Table 6). The growth of the fungal culture was best at 3% and yield of the aflatoxins was best at 5%.and production of aflatoxins, various nitrogen sources in various concentrations were used.

Table 6 Effect of peptone concentration on aflatoxin production by *A. flavus* (II) in YESPB[a] media.

Entry	Percentage of peptone	[b] Stationary culture		[c]Shake culture	
		[d]Mycelial dry wt.	[e]Crude aflatoxins	[d]Mycelial dry wt.	[e]Crude aflatoxins
1	Nil	0.12	0.112	0.07	0.100
2	1	0.18	0.152	0.11	0.152
3	2	1.28	0.170	1.22	0.170
4	3	1.54	0.173	1.54	0.173
5	4	1.49	0.248	1.49	0.230
6	5	1.52	0.245	1.52	0.236

[a] YESB - Yeast Extract Sabouraud Broth.
[b] In incubator.
[c] In rotatory shaker at 150rpm.
[d] Weight of dry mycelia was represented in g/100 mL.
[e] Weight of crude aflatoxins was reported in g/100 mL.

Extraction of aflatoxins using various solvents

In order to study the best solvent for extraction of aflatoxins, the experiment was performed using various polar and non-polar solvents viz. methanol, ethanol, propanol, chloroform, dichloroform and acetonitrile under similar conditions (Figure 4). It was observed that chloroform was the best suitable solvent for obtaining maximum yield.

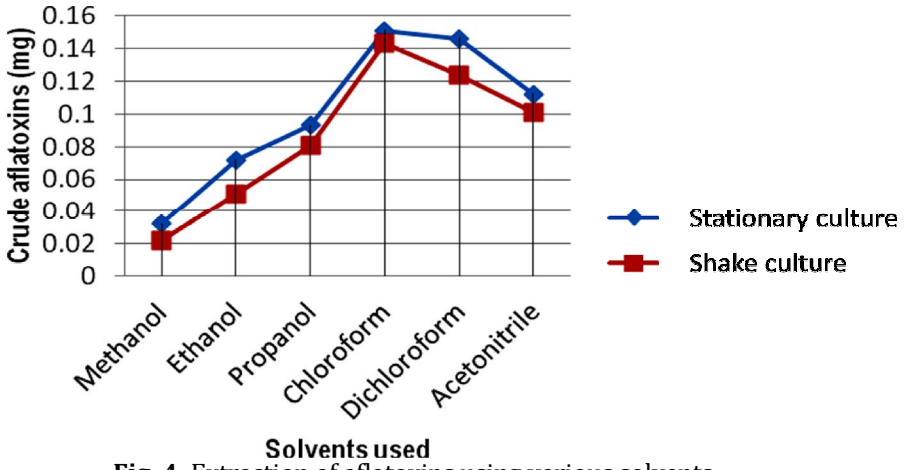


Fig. 4. Extraction of aflatoxins using various solvents

Purification and characterization of aflatoxins

Purification of crude aflatoxin toxin was done by column chromatography it includes column preparation, capturing of the aflatoxins and washing was done following the Aflaprep® (R-Biopharm) procedure as a reference standard with some modification. Any aflatoxin which is present in the sample is retained by the antibody within the gel suspension. The column is washed with water to remove extraneous non-specific material. The bound toxin is released by the antibody following elution from the column with methanol. The preliminary detection of crude aflatoxin was done by TLC and further detection and characterization of purified aflatoxin was done by UV-visible spectrophotometer and Fourier Transformer Infrared spectrophotometer respectively.

DISCUSSION

Basal medium containing sucrose, peptone, yeast extract at stationary culture / shake culture for the production of *A. flavus* (II). For the growth of fungi and aflatoxins production pH and temperature is an important factor. The best result observed at pH 6 and suitable temperature was found to be 30^{O}C in an incubator. The influence of sucrose concentration on aflatoxin production was found to maximum at 20 and 25 % sucrose respectively in YESPB medium and observed that 25% sucrose as a carbon source was suitable for aflatoxin production. The effect of peptone concentration on aflatoxin production by *A. flavus* was obtained maximum at 5 % concentration of peptone was much higher than those normally used in fungal growth media. Therefore YESPB medium of 25% sucrose, 5% peptone and 2% yeast extract apparently provided all necessary ingredients for the production of high level of aflatoxins. The YESPB medium is easy to prepare, relatively inexpensive and suitable for high yield of aflatoxins than those reported for other media as it contain extra nitrogen source peptone beside yeast extract which is helpful in aflatoxin production. For this reason, YESPB medium appears to be suitable for both production and screening fungi for their ability to produce aflatoxins for various research studies. The results (Table 2-6) show that, *A. flavus* (II) obtained from groundnuts produced aflatoxins B_1. The yield of aflatoxins was found to be maximum, when growth of fungal cultures was carried out in stationary conditions incubated at 28±2^{O}C, the same experiment was performed using rotatory shaker the yield of the mycelia was found maximum but yield of aflatoxins was decreased which indicates that the production of aflatoxins in stationary conditions is more suitable. The extraction of aflatoxins was found to maximum by chloroform and minimum in methanol under the same conditions, yield of aflatoxins decreases in the order as butanol, propanol, acetonitrile and ethanol were used for extraction this is due to the fact that the solubility of the aflatoxins decreases in above the solvents.

TLC and UV spectrophotometer were also used for confirmation of aflatoxin B_1 in the extracts of *Aspergillus flavus* isolated from groundnut sample [14]. UV spectra of aflatoxin B_1 shows characteristic peak at 425 nm (Fig.6). Fourier transform infrared (FTIR) spectroscopy is a rapid, easy, and convenient

analytical method for determining aflatoxins. The aflatoxins exhibit characteristic absorption bands at wavelengths 3004- 2969 cm^{-1} for CH_2, aromatic =CH, –C–H, C=C and phenyls, [15] 1744-1720 cm^{-1} for C=O [16]. 1364-1369 cm^{-1} for methyl adjacent to epoxy ring, 1217-1220 cm^{-1} for in-plane –CH bending of phenyl 1035-1037 cm^{-1}) for symmetric stretching of =C–O–C or symmetric bending of phenyl. In FTIR spectra similar peaks were observed in samples isolated from toxigenic isolate as shown in Figure 7.

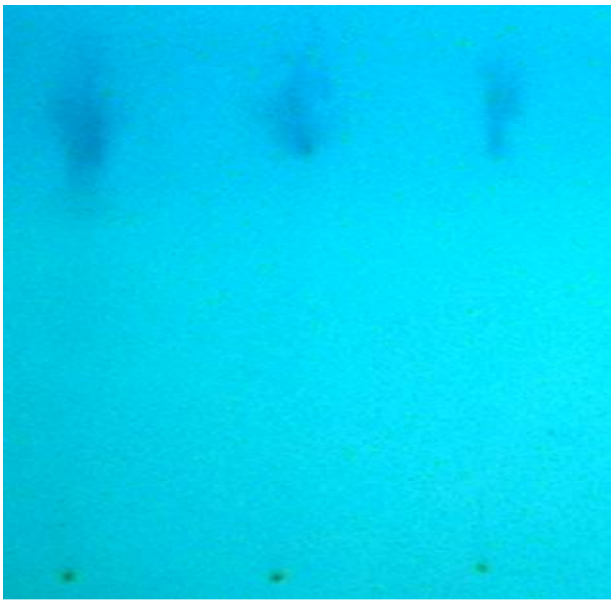


Fig 5. TLC plate observed under UV Light

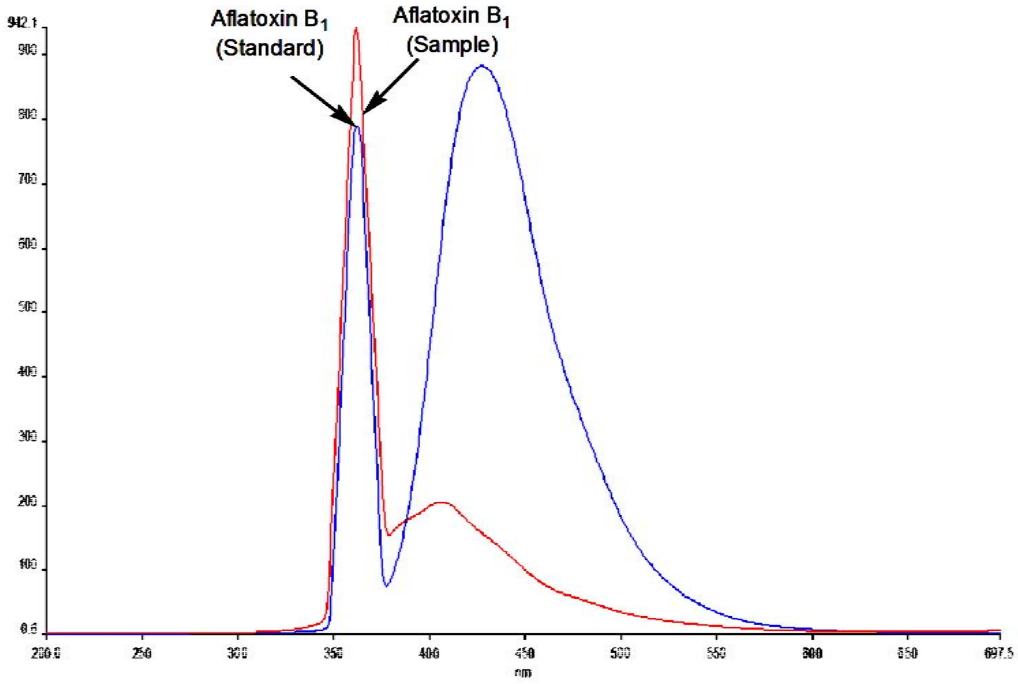


Fig. 6. UV-visible spectra of standard and isolated aflatoxin B_1

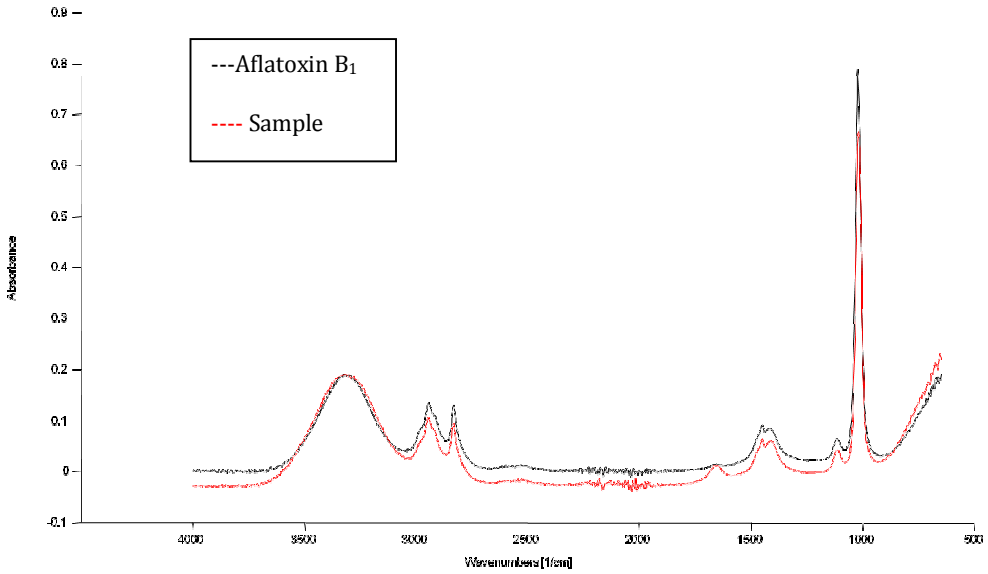


Fig. 7. FT-IR spectra of standard and isolated aflatoxin B_1

CONCLUSIONS

In conclusion, groundnuts samples were collected and screened for *A. flavus* which was identified and characterized morphologically by carbohydrate assimilation test. The isolated *A. flavus* (II) culture was used for aflatoxins production and optimized different physical parameter. Detection and characterization was done by TLC, UV-Vis spectroscopy and FT-IR spectroscopy respectively.

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