Pak. J. Agri. Sci., Vol. 58(2), 447-460; 2021
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/21.765
http://www.pakjas.com.pk

EVALUATION OF GROWTH MEDIA AND *IN-VITRO* CONDITIONS FOR THE ASSESSMENT OF *Aspergillus flavus* GROWTH AND AFLATOXIN B1 IN GRAPES

Muhammad Humza[1], Li Li[1,†], Gang Wang[1], Shujian Huang[2], Mingjun He[2], Chengrong Nie[2], Yongquan Zheng[3], Babar Iqbal[4], Fuguo Xing [1,*] and Yang Liu[1,2,*]

[1]Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences/Key Laboratory of Agro-products Quality and Safety Control in storage and Transport Process, Ministry of Agriculture and Rural Affairs, Beijing 100193, P.R. China; [2]School of Food Science and Engineering, Foshan University, Foshan 528231, P.R. China; [3]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, P.R. China; [4]Plant Pathology Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan

[*]Corresponding author's e-mail: liuyang@fosu.edu.cn; xingfuguo@caas.cn
†Equally contributed as first author

Aflatoxin B_1 (AFB_1) is one of the toxic secondary metabolites of fungi belonging to the genus *Aspergillus*. AFB_1 causes hepatotoxicity and carcinogenicity. Mostly, cereals, nuts, fruits, and stored commodities are prone to their attack. It has great health and economic importance. Under suitable conditions, fungus shows maximum pathogenicity which leads to the abundant production of AFB_1. This study encompasses the selection of the most pathogenic strain producing abundant AFB_1. For this purpose, 50 isolates were tested and YF18 was the most pathogenic. This isolate was then evaluated on 15 culture media which concluded that Yeast Extract Sucrose (YES) agar and Yeast Peptone Dextrose (YPD) agar media proved best for the optimal growth of *A. flavus* and abundant yield of AFB_1. Moreover, the culture media showing optimal growth under different levels of growth conditions i.e., temperature, pH, and water activity, and their effect was verified with the aid of correlation and response surface methodology. The culture media were checked at five levels of temperature (15ºC, 20ºC, 25ºC, 30ºC, and 35ºC), pH (3.5, 4.0, 4.5, 5.0 and 5.5), and water activity (0.80, 0.85, 0.90, 0.95, and 0.995) were tested, confirming that temperature of 30ºC, pH of 5.5 and water activity level of 0.995 significantly enhanced the growth of *A. flavus* and production of AFB_1 ($P<0.05$); while correlation, as well as response surface methodology, also concluded the level of conditions appropriate for mycelial growth and AFB_1 yield. Afterward, the isolate YF18 was applied to the grapes and biochemical parameters were evaluated. The biochemical analysis concluded that fungal infected grapes showed less acidity, TSS, total sugars, TPC, and DPPH as compared to non-treated grapes while the berry size and berry weight was also reduced in infected grapes. The isolate was subjected to 16S rDNA analysis for identification which revealed that fungus is *A. flavus* and the aflR gene was responsible for activation and release of aflatoxin B_1. The present study will help to devise management strategies to reduce the incidence of fungus and its respective toxin.

Keywords: *Aspergillus flavus*; Aflatoxin B_1; culture media; growth conditions; mycelial growth; AFB_1 yield.

INTRODUCTION

AFB_1 is one of the toxic substances produced as a secondary metabolite by a genus of filamentous fungi named *Aspergillus* (Giray *et al.*, 2007). Around 20 species of *Aspergillus* are reported to produce aflatoxins including *A. flavus*, *A. parasiticus*, and *A. nominus*. *A. flavus* is the fungus responsible for causing Aflatoxin B_1 toxicity (Payne and Brown, 1998; Saleemullah *et al.*, 2006) in cereals including wheat, rice, maize; nuts including walnuts, peanuts, and cotton (Severns *et al*., 2003). AFB_1 is hepatotoxic, carcinogenic, and teratogenic by nature infecting humans and animals either directly or indirectly (Vaamonde *et al.*, 2003). Food or feedstuff contaminated with AFB_1 can cause poisoning of the respiratory system leading to irritation under severe cases (Varga *et al.*, 2011).

A. flavus is the main agent which produces AFB_1 in contaminated product and when consumed in excess causes aspergillosis (Saldan *et al.*, 2018). *A. flavus* produces four aflatoxins namely B_1, B_2, G_1, and G_2. Apart from these toxins, *A. flavus* can also produce other toxins such as tenuazonic acid, nitropropionic acid, and cyclopiazonic acid (Atehnkeng *et al.*, 2008; Probst *et al.*, 2014). Since its discovery, it has been considered that *A. flavus* can only produce AFB_1 and

AFB_2 but with the passage of time and advancement in technology further strains were found to also produce AFG_1 and AFG_2 (Park and Bullerman, 1983). *A .flavus* produces globose to sub-globose type sclerotia which range in size from 400–700µm and the strains producing small sclerotia are mostly responsible for producing AFB_1 (Medina *et al.*, 2006). AFB_1 production is mostly dependent on certain environmental conditions such as temperature, pH and relative humidity. The conditions that are favorable for the production of AFB_1 are more strict than fungal growth (Nielsen *et al.*, 1989; Cairns-Fuller *et al.*, 2005). For the production of AFB_1, substrate is the most important factor which manages its production. The presence of appropriate media leads to the successful growth of fungus followed by an abundant production of AFB1. Certain strains of *A. flavus* produce different levels of AFB_1 under the same media and culture conditions which confirms that production of AFB_1 is specie dependent (Gqaleni *et al.*, 1997; Kumar *et al.*, 2017).

The activity of *A. flavus* and AFB_1 also have an impact on the biochemical and physiological properties of the grapes. Considerable efforts have been made to avoid the biochemical changes and keep the integrity of the fruit intact (Costa *et al.*, 2009; Wang and Wang, 2009; Fang *et al.*, 2011). Thus, the effect of toxin-producing fungi and their toxins on fruit biochemical components is drastic. The genetic studies also proved to be an excellent tool for molecular characterization of fungus and also check the mechanism of toxin production and release by the fungal genome.

Keeping in view the above-mentioned facts, different growth media and culture conditions including temperature, pH, and water activity which are conducive for the growth of *A. flavus* and AFB_1 production were tested. The relationship of all these conditions was also compared with fungal growth and toxin production along with their biochemical and genetic properties.

MATERIALS AND METHODS

***Sample Collection*:** The samples were collected from various vineyards in different grape-producing regions of China.

***Samples preparation*:** Collected grape samples were cut from branches and berries were surface sterilized and immersed in distilled water. The samples were then shaken in a shaking incubator at 28°C and 180 rpm for 60 minutes.

***Preservation of samples*:** After shaking the samples were preserved in 50% glycerol solution and stored at -80°C for further use.

***Screening of isolates*:** The collected samples after shaking were grown in Petri plates containing Potato Dextrose Agar (PDA) and then incubated at 28°C for obvious growth of the isolates.

***Separation of Isolates*:** Isolates grown showing more pathogenic growth were separated and added in PDB to maintain their growth.

***Sub-culturing of isolates*:** Isolates added in PDB were sub-cultured on PDA to purify and for further use.

***Preservation of isolates*:** After sub-culturing the isolates were preserved in 50% glycerol solution and stored in -80°C.

***Evaluation of culture media for growth of strain YF18*:** For the evaluation of growth on various media, mycelial growth was checked on every media using digital Vernier caliper to sort out the best growth media. Data were collected after 3, 5, and 7 days of inoculation. Fifteen growth media were used for this study viz. PDA, Water Agar, Czapeck Dox Agar, Martin Agar, Dichloran Rose Bengal Chlorophenicol (DRBC) Agar, Dichloran Glycerol 18 (DG-18), Yeast Peptone Dextrose (YPD) Agar, Yeast Extract Sucrose (YES) Agar, Minimal Media (MM), Malt Extract Agar (MEA), Malt Yeast 40% Glucose (MY40G) Agar, APFP Agar, Complete Agar, Soy Agar, and Saburaud Dextrose Agar. These media were prepared at a volume of 1000mL and were autoclaved. The isolates were inoculated on all culture media and incubated at 28°C.

***Evaluation of growth conditions on the growth of strain YF18 Evaluation of Temperature*:** For the determination of temperature appropriate for the activity of strain, five temperature levels i.e., 15°C, 20°C, 25°C, 30°C and 35°C were put under consideration. The media showing the best growth was put under these temperature ranges after inoculation. Data regarding mycelial diameter were collected after 3, 5, and 7 days.

***Evaluation of pH*:** To note the effect of pH on the growth of fungus and its secondary metabolite production, the growth media was adjusted to 5 pH levels i.e., 3.5, 4.0, 4.5, 5.0 and 5.5, respectively. These levels of pH were attained using 0.1N HCl. Data on the diameter of fungal mycelium were collected after 3, 5 and 7 days.

***Evaluation of Water Activity (a_w)*:** For testing the effect of water activity, five water activity levels viz. 0.80, 0.85, 0.90, 0.95, and 0.995, respectively, were assigned to check the growth of fungi and AFB_1 production. The assigned water activity levels were maintained using sterilized 100% glycerol. Data regarding mycelial diameter were collected after 3, 5, and 7 days.

***Estimation of AFB_1 content*:** The extraction of AFB_1 was carried out using Acetonitrile (Sigma-Aldrich, USA). The method developed by (Cairns-Fuller *et al.*, 2005) was applied with slight modifications. According to this method, 2mm agar disk containing fungal growth were collected from random areas in an agar plate then added in 50mL methanol and vortex at 110 rpm for 2 minutes. The samples were then filtered to collect in vials and subjected to HPLC (Waters, USA) for toxin estimation. Standard AFB_1 solution was used as a reference. Data were collected after 3, 5, and 7 days.

***Biochemical analysis acidity*:** Total acidity was measured according to the method in (AOAC, 2006). According to this method, 10mL of the sample was added toa 100mL flask and diluted up to 50mL using distilled water. Titration was done

against 0.1N NaOH and phenolphthalein indicator was used to check the point where the solution becomes pink in color. Acidity was determined using this equation:

$$\text{Acidity (as \% anhydrous CA)} = \frac{0.009 \times \text{volume of 0.1 N NaOH used}}{\text{Weight of the sample}} \times 100$$

***Total Soluble Solids*:** Total soluble solids were determined with the help of a hand refractometer (Eclipse, UK) according to the method described in (AOAC, 2006)

***Total Sugars*:** The method for determining total sugars is described in (AOAC, 2006). According to this method, 50mL filtrate was collected in a flask using a pipette. Citric acid was then added at the rate of 5g and 50mL distilled water was added. Then it was boiled and cooled. Phenolphthalein indicator was added to neutralize and then 20% NaOH solution was added till the solution becomes pink in color. Afterwards, 1N HCl was added dropwise to disappear the pink color. The pH was adjusted to 8.1 for performing titration. The following equation was used to determine total sugars:

$$\% \text{ Total Sugars} = \frac{\text{Fehling's solution factor} \times 100 \times \text{Dilution}}{\text{The volume of sample used} \times 1000}$$

The analysis for glucose, fructose, and sucrose was performed using the method described by (Cadet, 2003) in which the sugars were quantitatively analyzed.

***Total Phenolic Contents*:** According to (Ghafoor, 2009), the Folin-Ciocalteu method is widely used for the determination of total phenolic contents. Five-mL of Folin-Ciocalteu reagent was mixed with 1mL aliquots of 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mg/mL Catechol solutions in ethanol and 4mL sodium carbonate solution was added. The absorbance was measured at 765nm while the calibration curve was also plotted as a standard.

Each treatment was taken at a rate of 1mL and absorbance was measured after 1 hour of standard running. TPC was measured in gallic acid equivalents (GAE) by the following equation

$$C = \frac{c \times V}{m}$$

Where: C = total content of phenolic compounds extract (mg/g), in CAE; c = the concentration of Catechol calculated from the calibration curve (mg/mL); V = the volume of extract (mL); M = the weight of methanolic extract

***Radical Scavenging Activity (DPPH)*:** The radical scavenging activity was determined according to the method proposed by Ghafoor (2014) with slight modifications. Ethanol (10mL) was taken to dissolve dried extracts. DPPH solution was prepared in advance. Three-mL of DPPH solution was mixed in 77µL solution and samples were kept in dark for 15 minutes at room temperature and absorbance was measured at 515nm using UV/visible light spectrophotometer (CESIL CE7200).

The absorbance of a blank sample containing the same amount of ethanol and DPPH solution was also measured according to the method mentioned above. Radical scavenging activity was calculated by the following equation

$$\text{Reduction of absorbance (\%)} = \frac{[(AB - AA)]}{AB} \times 100$$

Where: AB = absorbance of blank sample (t = 0 min); AA = absorbance of tested extract solution (t = 10 min)

***Identification of strain via 16S rDNA sequencing*:** For the identification of strain, 16S rDNA sequence analysis was performed. The 16S ribosomal DNA gene analysis was performed following E.Z.N.A DNA Kit (Omega Bio-tek, Inc.). For polymerase chain reaction (PCR), the amplification of DNA template was performed using a universal primer set. PCR was performed in a reaction mixture containing 1µL of universal primer, 9.5µL of de-ionic water and 12.5µL of Go-Taq polymerase and 1ng/µL of template DNA making a total volume of 25µL (Susilowati *et al*., 2015). PCR was performed using a TaKaRa PCR Thermal Cycler Dice TP600 (Takara, Japan) under the following conditions: pre-denaturation (94°C for 5 minutes), denaturation (94°C for 30seconds), annealing (55°C for 30 seconds), extension (72°C for 1 minute), 35 cycles of the above procedure, final extension (72°C for 7 minutes), then preserve at 4°C. Amplified PCR products with a single fragment were confirmed through electrophoresis Smart view pro-1000 images system (Thmogran, MG major science, Taiwan) in 1% (w/v) agarose gels with 1 × TAE buffer containing 0.1 GelRed Nucleic Acid Stain (10000×, Biotium, USA) and photographed under UV light.

DNA sequencing was conducted by the lab of TSINGKE Biological Technology Company. Aligned sequences were compared to the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was generated base on 16S DNA genes including neighbor-joining related genes. MEGA version 6.0 software package was used to construct phylogenic tree following the procedure of multiple sequence alignments and comparisons were aligned by Clustal W software.

***Statistical Analysis*:** The data were subjected to statistical analysis in which analysis of variance was performed on all parameters and means were compared using Tukey's Honestly Significance Difference (HSD) test at 5% level of significance while the correlation was performed among all the variables using Pearson's correlation test (α=0.05). All the analysis was done using Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: USA). Response surface methodology was studied with the help of Box-Behnken Design using Minitab 17.

RESULTS

***Screening and Isolation of most pathogenic isolate*:** From the grape sample, around 50 isolates were screened out from

Table 1. Screening of isolates producing AFB_1.

Sr. No.	Isolate	Activity	Sr. No.	Isolate	Activity	Sr. No.	Isolate	Activity
1	T	+	22	C1921	+	43	VY72	+
2	UI	-	23	XW912	-	44	BP88	+
3	Y	++	24	YT13	-	45	VR39	+
4	Q2	+	25	YS45	-	46	XP98	-
5	W45	-	26	MQ45	-	47	ZE35	-
6	S21	-	27	YF11	-	48	ST11	-
7	**YF18**	+++++	28	ZE39	-	49	HP86	-
8	I12	++	29	JK18	++	50	MH20	++
9	P23	-	30	JY34	-			
10	BY61	+	31	BI28	-			
11	NB34	-	32	ZW17	-			
12	BV12	+	33	CV61	-			
13	MI21	++	34	BI54	-			
14	XW89	-	35	1S23	-			
15	SD09	+	36	2C4S	-			
16	FR10	-	37	OI12	-			
17	CZ45	-	38	PL67	-			
18	XQ78	-	39	MU95	++			
19	NR92	+	40	DE24	-			
20	MU39	-	41	YT97	-			
21	PL45	+	42	IM38	-			

"+": Less growth; "++": Moderate Growth; "+++++": Very Extensive Growth; "-": No growth

total. Strain YF18 of *A. flavus* found to be the most pathogenic as it showed extensive growth on grapes as well as on PDA. This strain was further checked under microscope for confirmation based on morphological examination and conidia structure. This strain was used for further evaluation.

Evaluation of culture media against YF18 and AFB_1: YES and YPD agar media were the most prominent growth media which displayed the highest mycelial growth of fungus as compared to others. While DRBC agar media showed less growth as compared to other tested media. During the estimation of AFB_1 content of *A. flavus* strain YF18, it was observed that YES and YPD agar media showed more concentration of AFB_1 while water agar media showed minimum and soy agar media showed no signs of AFB_1 as compared to other growth media (Figure 1). YES and YPD agar media were further investigated under various levels of temperature, pH, and water activity.

Effect of *in-vitro* conditions on the growth of isolate YF18and on AFB_1 concentration

***Temperature*:** During the evaluation of temperature, the trend of growth in both growth media i.e., YES and YPD agar media increased with every unit increase of temperature from 15ºC to 30ºC; after 30ºC growth started to slow down at a steady rate. Minimum growth was observed at 15ºC while

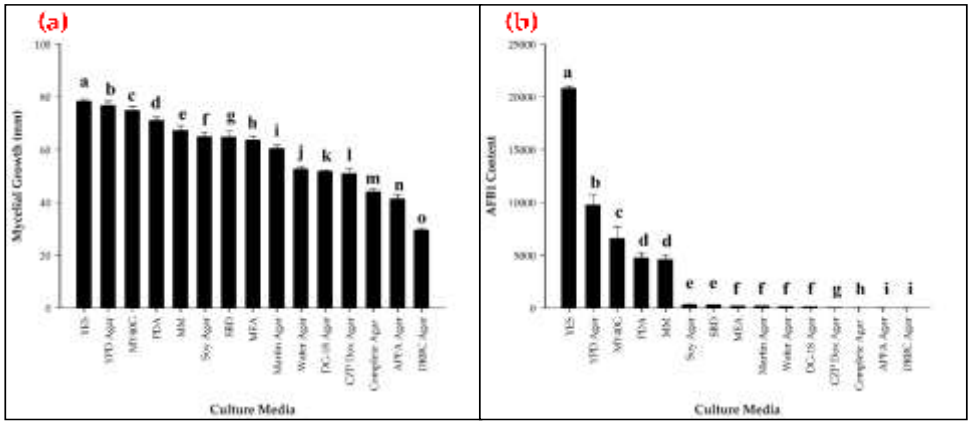

Figure 1. Evaluation of (a) mycelial growth and (b) AFB_1 content of isolate YF18 on different growth media. Error bars represent SE. Alphabetic letters represents statistical significance at $P \leq 0.05$

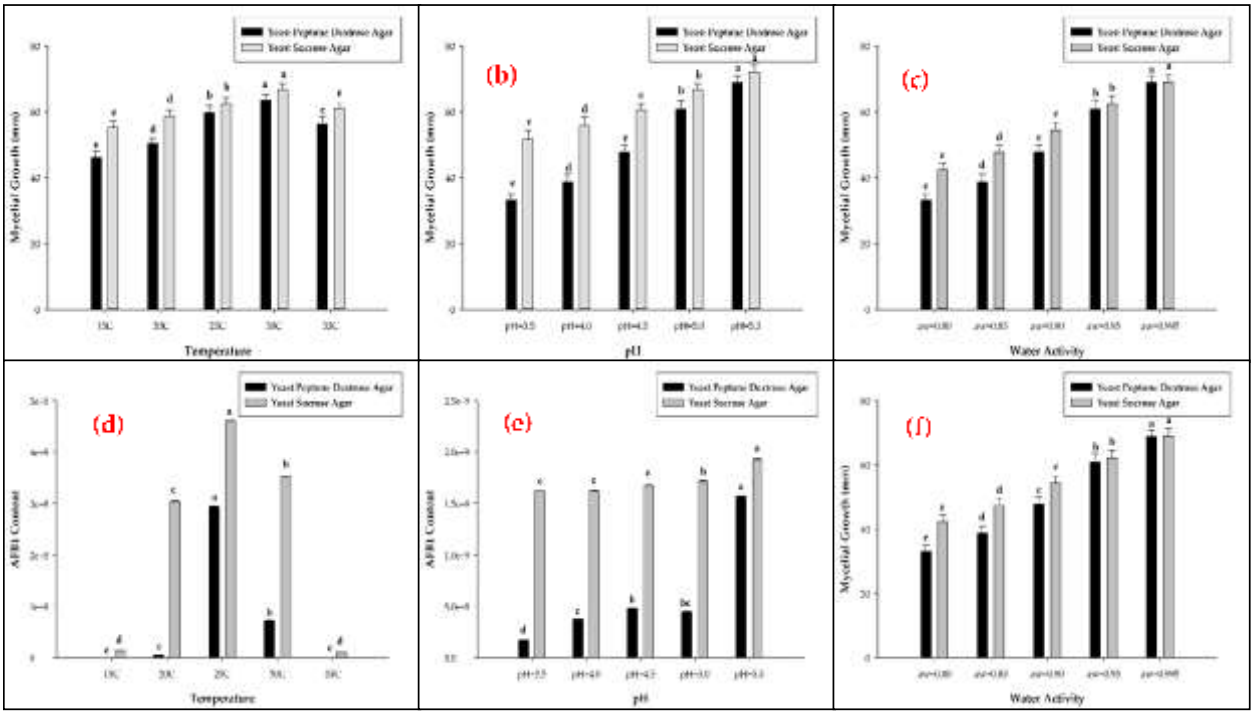

Figure 2. Evaluation of mycelial growth and AFB_1 content of isolate YF18 on Yeast Peptone Dextrose agar and Yeast Sucrose agar media at different levels of temperature (a, d), pH (b, e), and water activity (c, f). Error bars represent SE. Alphabetic letters represents statistical significance at $P \leq 0.05$.

maximum growth was observed at 30°C.

During the evaluation of various levels of temperature, it was observed that maximum content of AFB_1 was observed at 30°C; while, minimum AFB_1 content was found at 15°C. As the temperature increased every unit, the AFB1 content also increased till 30°C then declined. This trend was observed in both YPD and YES agar media ($P \leq 0.05$) (Figure 2).

***pH*:** With every unit increase in the level of pH, from pH 3.5 to 5.5 the mycelial growth increased. In both YES and YPD agar media, the selected isolate showed increased growth as the trend of growth increased it can be concluded that fungal strain may tolerate moderately acidic to highly acidic environment. Growth was minimum at pH 3.5 while maximum growth at pH 5.5.

Considering the pH on both culture media, maximum AFB_1 concentration was observed at pH 5.5 while minimum concentration was observed at pH 3.5. This displays an increasing trend of AFB_1 concentration concerning pH at 5.5. The decrease in acidic conditions helps the fungus to produce a maximum amount of AFB_1 in both culture media ($P \leq 0.05$) (Figure 2).

***Water Activity (a_w)*:** Water activity showed a directly proportional relationship with the growth of isolate as water activity increased, fungal growth also increased and vice versa. During the evaluation of water activity levels both in YES and YPD agar media, maximum growth was observed at 0.995 level of water activity while minimum growth of isolate was observed at 0.80 level of water activity.

Water activity increased rate of AFB_1at level of 0.995 while a decreased rate of AFB_1 production was at 0.80 level on both YES and YPD agar media. This trend in water activity illustrates that increased level of water activity enhances the production of AFB_1 and vice versa (Figure 2).

Correlation of in-vitro conditions with fungal growth and AFB_1 production: A significant relationship of temperature, pH, and water activity at all levels was observed with the growth of fungi and toxin production. All the factors equally contributed towards the growth of fungus and toxin production as shown in Table 2.

A significant and positive correlation with mycelial growth was observed at every level of temperature on both YES and YPD agar media. On YPD agar a positive correlation i.e., r=0.9553, 0.9566, 0.9630, 0.9714 and 0.9473 was observed at 15°C, 20°C, 25°C, 30°C and 35°C, respectively regarding mycelial growth at 5% level of significance. Likewise, on YES agar, a similar trend of correlation was observed between temperature ranges and fungal mycelial growth. The correlation between temperature ranges 15°C, 20°C, 25°C, 30°C, and 35°C and mycelial growth was 0.9695, 0.9697, 0.9763, 0.9778, and 0.9733, respectively. Similarly, regarding AFB_1 production on YPD agar, positive and significant correlation was observed i.e., r= 0.7646, 0.7664, 0.7773, 0.8932 and 0.7669 at 15°C, 20°C, 25°C, 30°C and 35°C,

Table 2. Correlation of Growth Conditions with isolate YF18 mycelial growth and AFB_1 production on Yeast Peptone Dextrose Agar & Yeast Sucrose Agar media.

Cultural Conditions			Isolate YF18			
			Yeast Peptone Dextrose Agar		Yeast Sucrose Agar	
			Mycelial Growth	AFB_1 Production	Mycelial Growth	AFB_1 Production
Temperature	**15ºC**	*r value*	0.9553*	0.7646*	0.9695*	0.7646*
		p value	0.0190	0.0450	0.0170	0.0440
	20ºC	*r value*	0.9566*	0.7664*	0.9697*	0.7652*
		p value	0.0180	0.0490	0.0150	0.0440
	25ºC	*r value*	0.9630*	0.7773*	0.9763*	0.8971*
		p value	0.0170	0.0430	0.0130	0.0290
	30ºC	*r value*	0.9714*	0.8932*	0.9778*	0.9556*
		p value	0.0150	0.0290	0.0140	0.0180
	35ºC	*r value*	0.9473*	0.7669*	0.9733*	0.7648*
		p value	0.0270	0.0440	0.0140*	0.0460
pH	**3.5**	*r value*	0.9629*	0.6059*	0.9745*	0.6036*
		p value	0.0160	0.0160	0.0110	0.0500
	4.0	*r value*	0.9673*	0.7645*	0.9787*	0.7646*
		p value	0.0170	0.0440	0.0130	0.0490
	4.5	*r value*	0.9791*	0.7648*	0.9891**	0.7649*
		p value	0.0130	0.0470	0.0030	0.0460
	5.0	*r value*	0.9802*	0.9237*	0.9905	0.9578*
		p value	0.0120	0.0250	0.0230*	0.0430
	5.5	*r value*	0.9898**	0.9496**	0.9917**	0.9662**
		p value	0.0090	0.0020	0.0070	0.0010
Water Activity (a_w)	**0.80**	*r value*	0.9629*	0.7560*	0.9739*	0.7645*
		p value	0.0160	0.0450	0.0140	0.0460
	0.85	*r value*	0.9629*	0.7640*	0.9781*	0.7646*
		p value	0.0120	0.0440	0.0130	0.0450
	0.90	*r value*	0.9791*	0.7645*	0.9814*	0.7650*
		p value	0.0130	0.0460	0.0120	0.0460
	0.95	*r value*	0.9873*	0.7645*	0.9836*	0.7654*
		p value	0.0170	0.0490	0.0110	0.0490
	0.995	*r value*	0.9898**	0.8647*	0.9853**	0.9193*
		p value	0.0090	0.0450	0.0010	0.0250

*: Significant at P≤0.05 **: Significant at P≤0.01

respectively while positive and significant correlation was also observed for YES agar i.e., r= 0.7646, 0.7652, 0.8971, 0.9556 and 0.7648 at 15ºC, 20ºC, 25ºC, 30ºC and 35ºC, respectively.

Considering the pH factor, the correlation was found to be significant and positive as with every level increase in pH on both YPD and YES agar media. In case of mycelial growth on YPD agar media, correlation values were 0.9629, 0.9673, 0.9791, 0.9802 and 0.9898 for pH 3.5, 4.0, 4.5, 5.0 and 5.5, respectively, while for YES agar, correlation values at pH 3.5, 4.0, 4.5, 5.0 and 5.5 were 0.9745, 0.9787, 0.9891, 0.9905 and 0.9917, respectively. Considering the toxin production similar trend was observed as in YPD agar i.e., significant and positive correlation was found at all levels of pH i.e., r=0.6059, 0.7645, 0.7648, 0.9237 and 0.9496, respectively while for YES agar, positive and significant correlation was observed i.e., r= 0.6036, 0.7646, 0.7649, 0.9578 and 0.9662 for pH 3.5, 4.0, 4.5, 5.0 and 5.5, respectively.

For relationship of water activity with YF18 mycelial growth, it was observed that on YPD agar at 0.80, 0.85, 0.90, 0.95 and 0.995 levels of water activity correlation values were positive and significant i.e., r= 0.9629, 0.9629, 0.9791, 0.9873 and 0.9898, respectively, while on YES agar at 0.80, 0.85, 0.90, 0.95 and 0.995 levels of water activity correlation values were also positive and significant i.e., r= 0.9739, 0.9781, 0.9814, 0.9836 and 0.9853, respectively at 5% level of significance. In case of AFB_1 production, significant and positive correlation was observed i.e., r= 0.7560, 0.7640, 0.7645, 0.7645 and 0.8647 on YPD agar at 0.80, 0.85, 0.90, 0.95 and 0.995 levels of water activity, while on YES agar at 0.80, 0.85, 0.90, 0.95 and 0.995 levels of water activity, r values were 0.7645, 0.7646, 0.7650, 0.7654 and 0.9193, respectively showing significant and positive correlation.

Optimization of growth parameters for mycelial growth and AFB₁ yield: The cultural conditions for the mycelial growth of YF18 and toxin concentration were assessed based on response surface methodology via Box Behnken design. The coding of variables is shown in Table 3. The data was applied to the following equation:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_{ii} X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ii} X_i X_j + \varepsilon$$

where "*Y"* was the response variable, "β_0" was the intercept constant, "*βi"*, "*βii"*, "*βij"* were the regression coefficients of "X_1", "X_2", "X_3", "X_i", "X_j" were coded values of independent variables and "ε" is an error term.

Table 3. Variables defined for analysis under Box-Behnken Design.

Factor	Coded Symbols	Levels		
		–1	0	1
Temperature (°C)	X_1	15	25	35
pH	X_2	3.5	4.5	5.5
Water Activity (a_w)	X_3	0.80	0.90	0.995

***Yeast Peptone Dextrose Agar*:** The regression equation obtained from the analysis describes the extent of the relationship between temperature, pH, and water activity with mycelial growth and toxin content on YPD agar is as follows:

Y (Mycelial Growth) = 276 + 4.25 X_1 + 18.8 X_2 + 584 X_3 + 0.0748 $(X_1)^2$ + 4.07 $(X_2)^2$ + 428 $(X_3)^2$ + 79.9 X_1X_2 + 2.48 × 10^3 X_1X_3 + 1.09 × 10^4 X_2X_3

R^2 value = 0.92; R^2 (pred.) value = 0.82

Y (AFB_1 Content) = 6.79 × 10^9 + 1.39 × 10^8 X_1 + 2.95 × 10^9 X_2 + 6.25 × 10^8 X_3 + 2.78 × 10^6 $(X_1)^2$ + 4.05 × 10^8 $(X_2)^2$ + 3.60 × 10^9 $(X_3)^2$ + 4.11 × 10^{16} X_1X_2 + 8.71 × 10^{17} X_1X_3 + 1.85 × 10^{19} X_2X_3

R^2 value = 0.99; R^2 (pred.) value = 0.99

Using ANOVA (Table 3, 4) for the model, it was found to be significant ($P \leq 0.05$). It exhibited that this model was adequate and reproducible due to a non-significant lack of fit ($P > 0.05$). The mycelial growth and toxin yield predicted by the above two regression equations were close to the observed ones (R^2 = 0.92, R^2 = 0.99). Based on the ANOVA, three independent variables had a significant effect on mycelial growth and toxin concentration. The above parameters for mycelial growth and toxin content were evaluated and concluded that optimum mycelial growth and toxin concentration could be achieved at a temperature of 30°C, a pH value of 5.5, and a water activity of 0.995. The zones of optimization are shown in the surface plots to illustrate the effects of factors on the response (Figure 3). This describes that fungus can survive under normal to slightly high temperature, with slightly acidic to normal pH and appropriate water content. In Table 6, run 12 having a combination of temperature at 25°C (coded value = 0), pH at 5.5 (coded value = 1) and water activity at 0.995 (coded value = 1) showed maximum mycelial growth and AFB_1 yield on both YPD agar and YES agar, respectively.

***Yeast Sucrose Agar*:** The regression equation obtained from the analysis describes the extent of the relationship between temperature, pH, and water activity with mycelial growth and toxin content on YES agar is as follows:

Y (Mycelial Growth) = 363 + 3.84 X_1 + 22.1 X_2 + 830 X_3 + 0.071 $(X_1)^2$ + 1.33 $(X_2)^2$ + 538 $(X_3)^2$ + 84.864 X_1X_2 + 3187.2 X_1X_3 + 18343 X_2X_3

R^2 value = 0.93; R^2 (pred.) value = 0.85

Y (AFB_1 Content) = 3.96 × 10^9 + 2.25 × 10^8 X_1 + 2.78 × 10^9 X_2 + 2.72 × 10^9 X_3 + 4.51 × 10^6 $(X_1)^2$ + 3.87 × 10^8 $(X_2)^2$ + 1.63 × 10^9 $(X_3)^2$ + 6.28 × 10^{17} X_1X_2 – 6.14 × 10^{17} X_1X_3 + 7.58 × 10^{18} X_2X_3

R^2 value = 0.99; R^2 (pred.) = 0.95

Using ANOVA (Table 6, 7) for the model, it was found to be significant ($P \leq 0.05$). It exhibited that this model was also

Table 4. Analysis of Variance (ANOVA) for response surface model of mycelial growth by isolate YF18 on Yeast Peptone Dextrose Agar media

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
Model	9	7581169.00	842352.20	8581.42	0.027
X_1	1	206.86	206.86	2.11	0.026
X_2	1	2553.27	2553.27	26.01	0.004
X_3	1	2553.27	2553.27	26.01	0.004
$(X_1)^2$	1	206.86	206.86	2.11	0.026
$(X_2)^2$	1	61.31	61.31	0.62	0.045
$(X_3)^2$	1	61.01	61.01	0.62	0.046
$X_1 \times X_2$	1	528169.40	528169.40	2152.28	0.022
$X_1 \times X_3$	1	528169.40	528169.40	2152.28	0.021
$X_2 \times X_3$	1	6519188.00	6519188.00	26565.56	0.003
Error	5	490.79	98.16		
Lack-of-Fit	3	90.79	30.26	0.12	0.934
Pure Error	2	400.00	200.00		
Total	14	7581660.00			

R^2 = 0.92

Table 5. Analysis of Variance (ANOVA) for response surface model of aflatoxin B_1 content by isolate YF18 on Yeast Peptone Dextrose Agar media

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
Model	9	2.75×10^{35}	3.05×10^{34}	307.27	0.000
X_1	1	3.03×10^{9}	3.03×10^{9}	6.91	0.009
X_2	1	3.88×10^{18}	3.88×10^{18}	2212.47	0.000
X_3	1	3.59×10^{15}	3.59×10^{15}	2.05	0.012
$(X_1)^2$	1	2.87×10^{17}	2.87×10^{17}	163.50	0.000
$(X_2)^2$	1	6.07×10^{17}	6.07×10^{17}	346.17	0.000
$(X_3)^2$	1	4.32×10^{15}	4.32×10^{15}	2.47	0.017
$X_1 \times X_2$	1	1.18×10^{28}	1.18×10^{28}	2.69×10^{12}	0.032
$X_1 \times X_3$	1	1.09×10^{25}	1.09×10^{25}	2.48×10^{9}	0.021
$X_2 \times X_3$	1	1.40×10^{34}	1.40×10^{34}	3.18×10^{18}	0.016
Error	5	8.78×10^{15}	1.75×10^{15}		
Lack-of-Fit	3	3.00×10^{0}	1.00	2.28×10^{-16}	0.919
Pure Error	2	5.78×10^{15}	4.39×10^{15}		
Total	14	4.86×10^{15}			

$R^2 = 0.99$

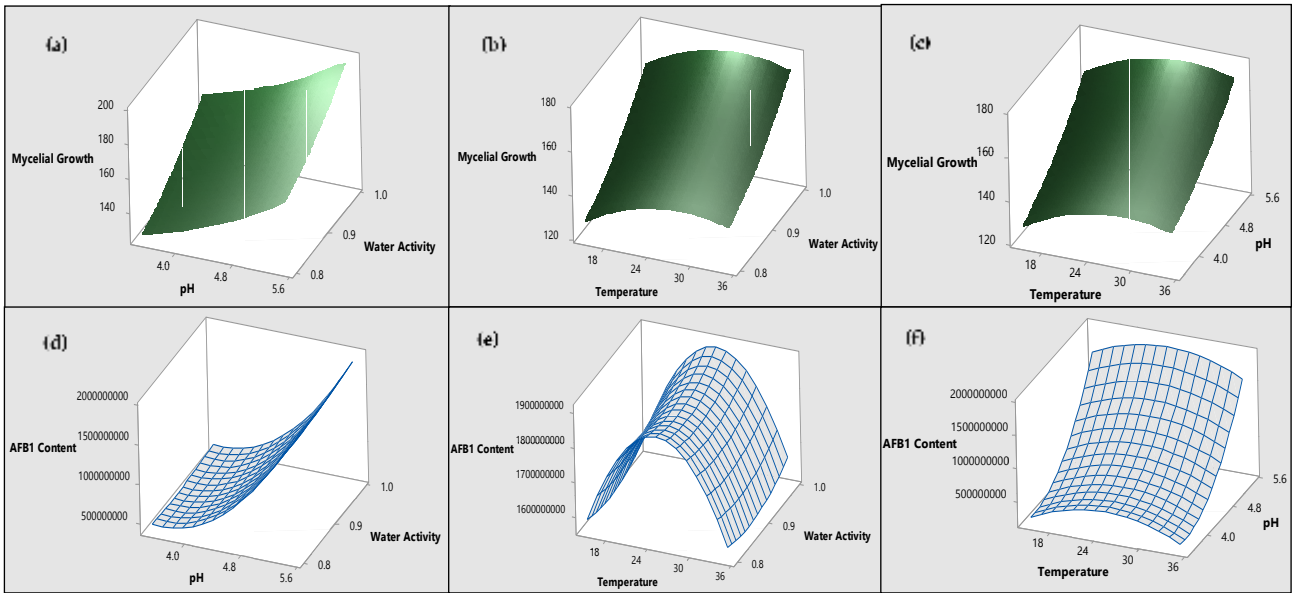

Figure 3. Response Surface plot for the effects of (a, d) pH and Water Activity (b, e) Temperature and Water Activity (c, f) Temperature and pH on mycelial growth and AFB_1 content, respectively of *A. flavus* (strain YF18) on yeast peptone dextrose agar media.

adequate and reproducible due to a non-significant lack of fit (*P>0.05*). The mycelial growth and toxin yield found by the above two regression equations were appropriate ($R^2 = 0.93$, $R^2 = 0.99$). Based on the ANOVA, three independent variables had a significant on mycelial growth and toxin concentration. The above parameters for mycelial growth and toxin content were found optimum showing a maximum mycelial growth and toxin concentration could be achieved at a temperature of 30°C, a pH value of 5.5, and a water activity of 0.995. The zones of optimization are shown in the surface plots to illustrate the effects of independent variables on the dependent variables (Figure 4). The surface plots also depict the optimized value of fungal behavior and toxin release.

***Biochemical analysis Acidity*:** As shown in Figure 5, the infected grapes showed less acidic behavior towards the infection with the mean value of 26.4% while healthy grape (control) showed more acidic behavior as the mean value was 42.8%. This behavior shows that fungal infection had a significant impact on the acidic properties of the grapes as compared to non-treated grapes.

***Total Soluble Solids*:** Total soluble solid contents analysis revealed that infected grape showed less content of total soluble solids (mean value = 80.79) while the control showed

Table 6. Analysis of Variance (ANOVA) for response surface model of mycelial growth by *A. flavus* (strain YF18) on Yeast Sucrose Agar media.

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
Model	9	13112400.00	145693.30	3993.78	0.017
X_1	1	66.59	66.59	1.83	0.035
X_2	1	817.70	817.70	22.42	0.005
X_3	1	1418.31	1418.31	38.88	0.002
$(X_1)^2$	1	186.30	186.30	5.11	0.037
$(X_2)^2$	1	6.51	6.51	0.18	0.049
$(X_3)^2$	1	96.67	96.67	2.65	0.046
$X_1 \times X_2$	1	54450.64	54450.64	597.11	0.037
$X_1 \times X_3$	1	94445.26	94445.26	1035.70	0.026
$X_2 \times X_3$	1	11597520.00	1159752.00	12717.97	0.011
Residuals	5	182.39	36.48		
Lack-of-Fit	3	82.39	27.64	0.303	0.872
Pure Error	2	100.00	50.00		
Total	14	1311422.00			

$R^2 = 0.93$

Table 7. Analysis of Variance (ANOVA) for response surface model of aflatoxin B1 content by *A. flavus* (strain YF18) on Yeast Sucrose Agar media.

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
Model	9	3.79×10^{35}	4.21×10^{34}	2.18×10^{19}	0.000
X_1	1	3.81×10^{13}	3.81×10^{13}	0.02	0.044
X_2	1	3.88×10^{18}	3.88×10^{18}	2005.33	0.000
X_3	1	3.59×10^{15}	3.59×10^{15}	1.86	0.031
$(X_1)^2$	1	7.52×10^{17}	7.52×10^{17}	388.45	0.000
$(X_2)^2$	1	5.53×10^{17}	5.53×10^{17}	285.55	0.000
$(X_3)^2$	1	8.95×10^{14}	8.95×10^{14}	0.46	0.027
$X_1 \times X_2$	1	1.48×10^{32}	1.48×10^{32}	3.06×10^{16}	0.041
$X_1 \times X_3$	1	1.37×10^{29}	1.37×10^{29}	2.83×10^{13}	0.033
$X_2 \times X_3$	1	1.40×10^{34}	1.40×10^{34}	2.88×10^{18}	0.019
Residuals	5	9.68×10^{15}	1.94×10^{15}		
Lack-of-Fit	3	0.18	0.06	3.10×10^{-17}	0.827
Error	2	9.50×10^{15}	4.75×10^{15}		
Total	14	1.35×10^{36}			

$R^2 = 0.99$

more level of total soluble solids (mean value = 286.64) which concludes that fungal infection significantly reduces the number of soluble solids present inside the grapes (Figure 5).

***Total Sugars*:** Total sugars revealed that grapes infected with *A. flavus* showed less amount of sugars compared to non-treated ones. The mean value for the control group was 221.97 and for infected grapes was 107.68 (Figure 5).

Fructose: Fructose content was reduced as the fungal attack reduces the availability to grape tissues while in the control group fructose was freely available as shown in Figure 5. The mean fructose value was 98.03 for the control group while for the fungus treated group was 16.54.

***Glucose*:** Glucose concentration in infected grape was found to be minimum as compared to the control group. These results described that fungal infection significantly reduces the glucose level in grapes as shown in figure 5. The mean glucose level for infected grapes was 12.81 while for the control group was 94.57.

***Sucrose*:** Like fructose and glucose, sucrose content was found to be decreased upon *A. flavus* infection while untreated grapes showed more content as shown in Figure 5. Mean sucrose content in healthy grape was 10.06 while for infected ones was 1.54.

***Total Phenolic Contents*:** The total phenolic contents were found to be minimum in fungal infected grapes while in the control group the phenolic activity was maximum (Figure 5). TPC in infected grapes showed a mean value of 1004.02 while uninfected grapes showed a mean value of 3409.1.

***Radical Scavenging Activity (DPPH)*:** The Radical Scavenging Activity (DPPH) was found to be maximum in the control group while in infected grapes the DPPH rate was found to be less as shown in Figure 5. Mean DPPH in the

Table 8. Experimental and Predicted values of *A. flavus* (strain YF18) mycelial growth and AFB_1 content in Box–Behnken Design

Run	Independent Variables			Yeast Peptone Dextrose Agar				Yeast Sucrose Agar			
				Mycelial Growth		AFB_1 Content		Mycelial Growth		AFB_1 Content	
	Temperature (X_1)	pH (X_2)	Water Activity (X_3)	Observed value	Predicted Value	Observed value	Predicted Value	Observed value	Predicted Value	Observed value	Predicte Value
1	–1	–1	0	127.15	126.66	178598167.7	178598169	154.76	156.01	193066188.7	1930661
2	1	–1	0	137.32	135.22	178637118.2	178637119.7	160.53	162.03	188696979.3	1886969
3	–1	1	0	162.88	162.26	1572355743	1572355745	174.98	176.73	1586823764	1586823
4	1	1	0	173.05	172.54	1572394693	1572394695	180.75	182.75	1582454554	1582454
5	–1	0	–1	127.14	126.23	482769457.9	482769460.2	158	160.25	497237478.9	4972374
6	1	0	–1	137.31	136.62	482808408.4	482808410.9	163.77	166.27	492868269.6	4928682
7	–1	0	1	162.87	161.56	525164362.1	525164364.9	184.63	187.38	539632383.1	5396323
8	1	0	1	173.04	172.23	525203312.7	525203315.7	190.4	193.4	535263173.8	5352631
9	0	–1	–1	125.92	126.54	470515572.7	470515576	156.55	159.8	636723012.2	6367230
10	0	1	–1	161.65	162.78	1864273148	1864273152	176.77	180.27	2030480587	2030480
11	0	–1	1	161.65	160.12	512910476.9	512910480.7	183.18	186.93	679117916.4	6791179
12	0	1	1	197.38	198.88	1906668052	1906668056	203.4	207.4	2072875492	2072875
13	0	0	0	137.06	136.76	672513669.1	672513673.4	166.15	170.4	885905505.1	8859055
14	0	0	0	168.25	169.99	793610507.8	793610512.3	185.16	189.66	1024670688	1024670
15	0	0	0	155.22	156.73	779664271.8	779664276.6	177.25	182	945871711.3	9458717

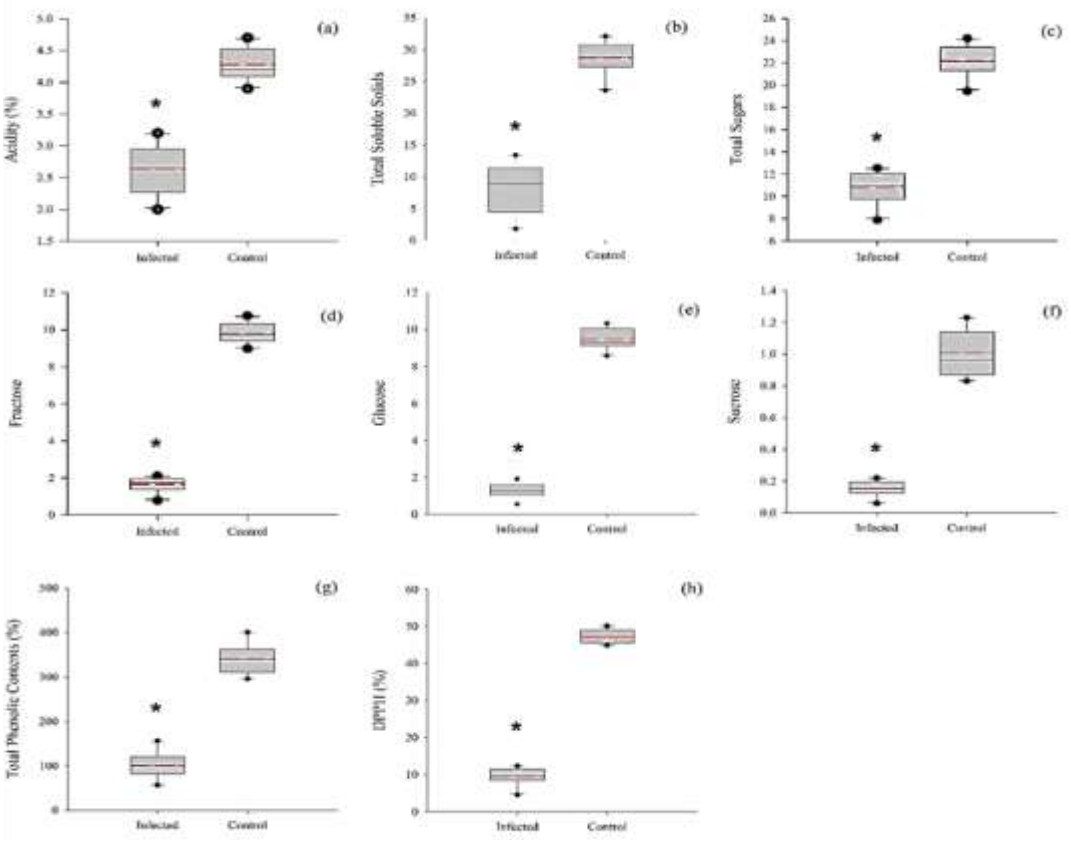

Figure 5. Comparative biochemical analysis of infected and healthy grape (a) Acidity (b) Total Soluble Solids (c) Total Sugars (d) Fructose (e) Glucose (f) Sucrose (g) Total Phenolic Contents and (h) DPPH. Asterisk (*) indicates the statistical difference at $P \leq 0.05$.

infected group was 95.08 while in the control group mean value was 473.33.

100-berries weight*:** As shown in Figure 6, the control group of grape berries showed more weight than the infected one. The infected group showed a mean weight of 84.3g while the control group showed a mean weight of 651g. Infection of *A. flavus* reduces the availability of necessary nutrients which reduces the vigor of grape berries.Grape berry diameter*:** As shown in Figure 6, the control group of grape berries showed more diameter than infected one. Infected group showed a

mean diameter of 3.27mm while control group showed mean diameter of 19.67mm. Infection of *A. flavus* makes the berries flaccid.

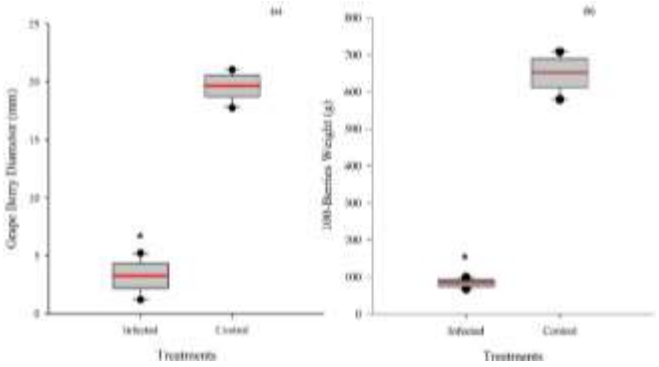

Figure 6. Comparative analysis of infected and healthy grape (a) Grape berry diameter (mm) (b) 100-Berries weight. Asterisk (*) indicates the statistical difference at $P \leq 0.05$.

Identification of AFB$_1$toxin-producing gene from A. flavus: The strain after 16S rDNA analysis was confirmed to be *A. flavus* and it had maximum severity. In the NCBI database, the 16S rDNA sequence was subjected to BLAST which concluded that it has around 100% similarity with *A. flavus*. After alignment of all the similar sequences obtained from BLAST, a phylogenetic tree was constructed which is shown in Figure 7. From the phylogenetic analysis, it was confirmed that the aflR gene was found to be the actual cause of producing aflatoxin B1 in *A. flavus* and is involved in its biosynthesis and regulation of toxin. Its molecular function is DNA binding with transcription factor activity, and it is RNA-polymerase II specific and also consists of zinc-binding ions.

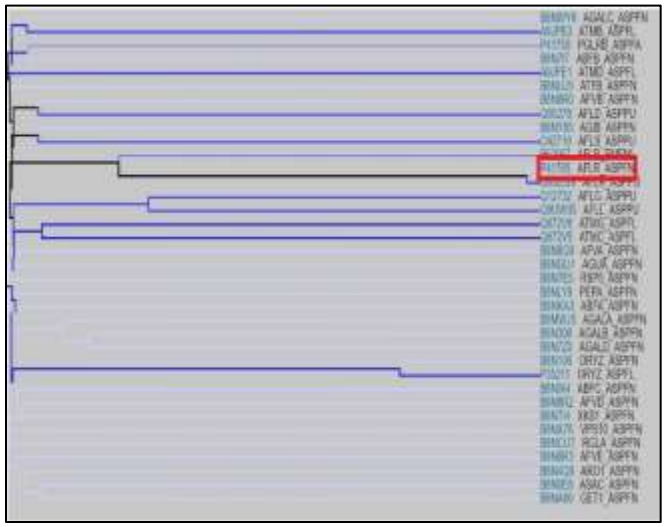

Figure 7. Phylogenetic analysis of *Aspergillus flavus* yielding various genes.

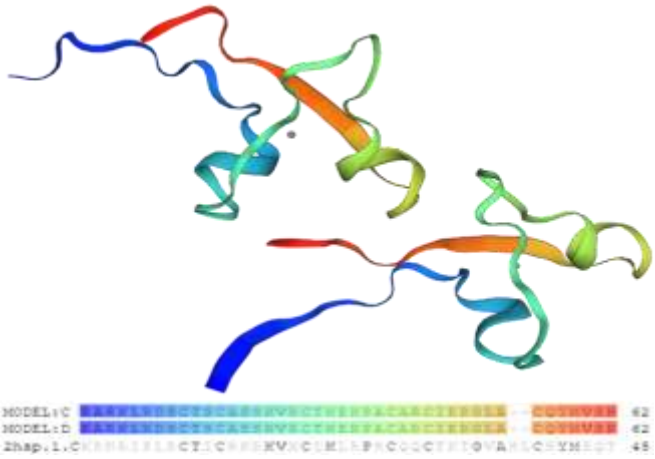

Figure 8. Structure of aflR protein

DISCUSSION

Mycotoxins are the toxic secondary metabolites produced as a result of fungal infection. With an increasing world population, the demand for food has also increased hence it also increases the risk of food security. Mycotoxins are also a threat to cereal grains, nuts, fruits, and stored products. Many methods are being under consideration that can propose new strategies for food security (Smith and Moss, 1985; Ritter *et al.*, 2011). Aflatoxin B1 is among the toxic mycotoxins which can cause toxicity in the blood, respiratory channels, and in severe cases death may also occur. Therefore, it is necessary to understand which conditions are suitable for the appropriate growth of fungus and toxin production (Pitt and Leistner, 1991; Coker, 1995; Gallo *et al.*, 2016).

Our study focuses on the evaluation of culture media and cultural conditions that promote fungal growth as well as the aflatoxin B1 production. For this purpose, fifteen culture media were checked with an *A. flavus* strain named YF18. The media that came under examination were PDA, water agar, Czapeck Dox agar, Martin agar, DRBC agar, DG-18 agar, YPD agar, YES agar, MM, MEA, MY40G agar, APFP agar, Complete agar, Soy agar, and Saburaud Dextrose agar. The results concluded that YES and YPD agar showed more growth of fungus and effective aflatoxin B_1 production among all culture media. These results showed resemblance with Gqaleni *et al.* (1997) who tested AFB_1 on CYA media and YES media and concluded that aflatoxin production was found to be maximum at YES media. Further, they also described that semisynthetic agars are also a very useful agent in aflatoxin studies. Park and Bullerman (1983) also suggested that the presence of a conducive substrate also favors the appropriate production of aflatoxin B_1. Frisvad (1981) also reported that YES agar is the most appropriate media for the production of AFB_1 and *A. flavus* mycelial growth.

Temperature of 30ºC, pH of 5.5, and water activity level of 0.995 were found to be optimum for the favorable growth of *A. flavus* (strain YF18) and abundant production of AFB_1. On YPD agar media, r value was 0.9714 for mycelial growth and 0.8932 for toxin production at 30ºC temperature. At pH 5.5, r values were 0.9898 and 0.9496 for mycelial growth and toxin production, respectively. At 0.995 level of water activity, r values were 0.9898 and 0.8647 for mycelial growth and toxin production, respectively. Similarly, on YES agar, r value was 0.9778 for mycelial growth and 0.9956 for toxin production at 30ºC temperature. At pH 5.5, r values were 0.9917 and 0.9662 for mycelial growth and toxin production, respectively. At 0.995 level of water activity, r values were 0.9853 and 0.9193 for mycelial growth and toxin production, respectively. Both culture media have shown an appropriate impact on mycelial growth and AFB_1 production at various levels of temperature, pH, and water activity, and their relationship was found to be significant and positive which depicts that fungus can adopt a wide range of cultural conditions to grow and produce toxin. These results are in acquaintance with Ritter *et al.* (2011) and Northolt *et al.* (1995) in which they concluded that temperature of 25ºC, pH of 5.2 showed maximum growth of *A. flavus* followed by AFB_1 yield.

Similarly, Gallo *et al.* (2016) reported that fungal growth and AFB_1were found to be maximum at 28ºC temperature and 0.96 water activity level. This level plays an important role in the abundant production of AFB_1 along with its gene expression. Gqaleni *et al.* (1997) concluded that temperature ranging between 32-35ºC and water activity of 0.95-0.996 also favors the abundant release of aflatoxin B_1. Northolt *et al.* (1977)described that various isolates of *A. flavus* can have a different temperature range of producing AFB_1. Every isolate works at best at its optimum temperature. Kheiralla *et al.* (1992) also observed that temperature 30ºC favors the aflatoxin B_1 production. Nielsen *et al.* (1989) observed that temperature ranging from 25-32ºC makes the fungus more efficient to produce AFB_1 while water activity around 0.98 to 0.99 is also very conducive for the *A. flavus*.

Determining the relationship of the cultural conditions with mycelial growth and toxin production it was found that all factors show positive and significant correlation at all levels of temperature, pH, and water activity. This is in agreement with previous studies (Ayerst,1969; Arora and Arora, 1991; Liu *et al.*, 2018). They also described that temperature and water activity are the critical factors that influence the production of aflatoxin B_1. Bacon *et al.* (1973) reported that the temperature of 30ºC along with 0.998 water activity level actively contribute to the synthesis of ochratoxin A.

It was found that temperature 30ºC with pH 5.5 and water activity around 0.995 was the most appropriate value for the optimum mycelial growth and toxin yield. These results show some acquaintance with Anjum *et al.* (1997) and Liu *et al.* (1999) in which they concluded that Box-Behnken Design is an efficient tool for determining the RSM for checking the optimization of various factors and conditions.

The biochemical properties of infected and non-infected grapes were checked, and it was concluded that infected grape berries showed less acidic properties, less soluble solid content, less concentration of sugars, less phenolic contents, and less radical scavenging activity while the control group showed maximum biochemical activities at 5% level of significance. This describes the efficiency of fungal strain under conducive conditions and the high availability of biochemical substances. Chen *et al.* (2019) concluded that AFB_1 causes the activation of novel enzymes which releases toxins and causes the changes in the chemical structure and process of grapes.

The isolate producing AFB_1 was confirmed *A. flavus* after 16S rDNA analysis which revealed that fungal gene aflR produces aflatoxin B1 and is involved in its biosynthesis. These results show some relationship with Chang (2003), Price *et al.* (2006) and Ehrlich (2009), who described that the aflR gene is responsible for AFB_1 biosynthesis in *A. flavus* and *A. parasiticus*.

***Conclusion*:** This study concludes that fungus can withstand at medium to high temperature, moderately acidic to the slightly acidic, and high content of water to produce aflatoxin B1. All these factors are positively correlated with fungal growth and AFB_1 production which shows a positive and significant relationship among these factors while the optimization via response surface methodology (RSM) also concludes that temperature 30ºC, pH 5.5, and water activity 0.995 shows more abundant AFB_1 production. Isolate YF18 was then checked concerning biochemical aspects concluding that fungal infected grapes showed minimum acidity, reduced TSS, decreased sugar content, and decreased radical scavenging activity. The 16S rDNA analysis confirmed that YF18 is *Aspergillus flavus* and the aflR gene is responsible for AFB_1 production and release. Determining the optimal condition, appropriate substrate, and pathogen virulence, a comprehensive strategy can be formulated to forecast the incidence of *A. flavus* and AFB_1 and regulate an appropriate management strategy against AFB_1.

Acknowledgment: We are thankful to the National Key Research and Development Program of China (2017YFC1600905) and (2019YFC1604502) for their support and funding of this research work.

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[Received 25 Jul 2020; Accepted 18 Dec 2020; Published (online) 18 April 2021]