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Innovation The Research Concept

Studies on effects of different pH, temperature and carbon sources on Mycelial growth and aflatoxins production by toxigenic strain of *Aspergillus parasiticus*.

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Abstract

Lyophilized culture of toxigenic strain of A. parasiticus NRRL-3000 was obtained from United States Department of Agriculture and was cultured and maintained on Potato Dextrose medium. Five different pH range 3.5, 4.5, 5.5, 6.5, 7.5 and six different temperatures 20, 24, 28, 32, 36 and 400C and six different carbon sources were used to observe their impacts on mycelial growth and aflatoxin production by *A. parasiticus (3000).* It was observed that among the pH 5.5, was most suitable as the highest dry mycelial weight 3.53 g/ 100 ml of culture and 3.7 mg aflatoxin, was obtained. Similarly at 28 C, dry mycelial weight 3.8g/ 100 ml of culture and 3.56 mg aflatoxin was elaborated. Among the carbon source glucose induced maximum mycelial growth i.e., 2.9 g / 100 ml culture and all the four aflatoxins among which B1 and G1 were elaborated 3.85 and 3.23 mg respectively. Rest of the pH, temperature and carbon sources had less promising impact on the above three parameters.

Key Words: *Aspergillus parasiticus*, *Aflatoxin*, Hydrogen ion concentration, Mycelial mat, Culture filtrate

Introduction

Aflatoxins are produced by toxigenic strains of fungi in the form of secondary metabolites and are responsible for development of disease among animals, birds and plants. Aspergilli are widely distributed. They have been isolated from many food stuff (Raper and Fennell 1965) different cereals, pulses and spices, and even from wine, form different part of world (Sargeant 1961; Allcroft and Carnaghan, 1962, 1963;Allcroft, 1965; and Uraguchi and Yamazaki 1978). In India production of aflatoxins have been reported by Rao et al; (1965), Dwarkanath et al; (1969), Mehan and Chauhan (1970); Tripathi (1973), Basappa and Sree Newas Murthy (1974), Mishra and Singh (1978); Bedi et al; (1981), Bilgrami et al (1980); Prasad (1982) and Roy et al (1988) from different states.

The aflatoxins constitute a number of structurally related secondary metabolites containing a coumarin ring fused to a bisdihydrofuran moiety with either a cyclopentenone ring-B series or a six member lactone-G_1 series. B and G indicate Blue and Green fluorescence of the chemical when viewed under UV radiation at 360 nm. Nesbitt *et al*; (1962) ,reported that aflatoxins can be separated chromatographically into B_1, B_2, G_1 and G_2.

A. parasiticus is a very potent producer of aflatoxins and which is very commonly associated with grains in India, has been less extensively investigated than *A. flavus*. Therefore, in the present work different climatic conditions such as pH , temperature and nutritional viz., different carbon sources have been used to observe its impact on the production of secondary metabolites i.e., the aflatoxins.

Materials and Methods

Preparation for mother culture: The surface of the vial was sterilized with ethanol soaked pre sterilized cotton. A file scratch was made on the sealed tube and it was re sterilized. The tube was broken and the pellet was taken out in a small beaker containing sterile distilled water in which the suspension of the pellet was prepared by manual shaking. This suspension of spores was streaked on PDA medium containing culture tubes and Petriplates and were cultured at 28± 1C. After 10^{th} day of incubation the cultured spores produced mycelial mat and number of spores. From these cultures spores were harvested in sterile distilled water. This was used for inoculation in liquid culture medium, having

different pH. When the pH was selected then on the same pH they were cultured on different temperature. When suitable temperature and pH were
selected then different carbon sources were used and the inoculated flasks were incubated at the above temperature.

Determination of the mycelial growth

10 days old cultures maintained at different pH were used for mycelial growth and aflatoxin elaboration. The mycelial mat was harvested by filtering the culture through Whatman filter paper No.-1, which was pre sterilized and weighed. The culture filtrates at different pH were preserved separately for the estimation of aflatoxins. The filters paper containing mycelial mat were placed in the desiccators. They were weighed next day and again placed inside the desiccators. This was repeated till constant weight was obtained. This was considered as dry mycelial weight Similar method was used for different temperatures and carbon sources.

Determination of aflatoxin concentration.

The method of Nebney and Nesbitt (1965) was followed for the extraction of aflatoxins, 5 ml of culture filtrate of 10 days old culture was taken in 100 ml separating funnel. 5 ml of chloroform (BDH) was added to it. The flask was shaken vigorously after closing the mouth with glass stopper for making a homogenous solution. Now this was allowed to separate two distinct phases appeared. Lower phase of chloroform containing dissolved aflatoxins was drawn off in a 50 ml beaker. To the upper portion again 5 ml chloroform was added again and the lower part was collected. This was repeated to extract the aflatoxin. Above chloroform containing dissolved aflatoxins was passed through sodium sulphate to remove trace of water. The sodium sulphate was again washed with the chloroform and all the chloroform were mixed together. The chloroform was evaporated after placing the beaker at 40^0C in the incubator. The residue was re dissolved in 1 ml chloroform. It was stored in a pre sterilized glass vial.

100 µl of the extract was loaded on glass plate coated with silica gel solution and activated at 100^0C for 1h. These spots were developed in a glass tank containing solution (Reddy et al; 1970) i.e., Toulene, Iso amyl alcohol and Methanol in 90:32:2 V/V. Plates were placed in the UV cabinet and based on the fluorescence B_1, B_2 and G_1, G_2 were marked. The Rf was determined as:-

Rf= Distance traveled by the solute / Distance traveled by the solvent.

Quantitative Estimation of Aflatoxins:

Positions of different aflatoxins were marked with pointed needle inside the UV cabinet at 365 nm. These bands were scrapped with a new blade and were added in a vial containing 3 ml cold methanol. All bands were collected separately, Methanol fraction was carefully decanded in a fresh vial. This was repeated and methanolic fractions were taken together in a fresh vial. It was centrifused at 5000 rpm for 5 minutes to separate trace of silica gel. UV absorption spectrum of the above methanolic solution was taken at 362 nm and then at 420 nm, using methanol as blank. Corrected optical density was calculated as

OD at 362 nm – OD at 420 nm.

The qualitative calculation was made by applying the formula proposed by Nebney and Nesbitt (1965).

$$DxMx10^6 / Єxlx1000 \quad µg / ml$$

Where D= Corrected OD.
M= Molecular Weight
Є= Molar extraction coefficient
l= light path of the cell

Here, value of D was obtained as mentioned above. Value of M&Є was used as proposed by Asao et al; (1965) and Hartley et al; (1963).

Result and Discussion:

The Rf value obtained for different aflatoxins has been presented in table-1.

pH denotes the degree of acidity and alkalinity. pH range of satisfactory growth of different fungi vary considerably. The mycelial growth and aflatoxin elaboration by *A. parasiticus* strain 3000 has been presented in table 2. From the table it is clear that highest dry mycelial weight was observed at pH 5.5 that was 3.8g/ 100 ml of culture medium. This was followed by 4.5 pH that was 3.2g/100 ml of culture medium. Lowest dry mycelial weight i.e., 1.02g/100 ml culture medium was obtained ats pH 7.5.

From the table it is also noted that production of aflatoxins was also maximum at 5.5 pH followed by 4.5 pH and lowest at 7.5 pH. Similarly among the four aflatoxins, B_1 was elaborated in higher amount followed by G_1. Both B_2 and G_2 were elaborated in traces, which were not calculated quantitatively.

Mycelial growth and aflatoxin production were also determined at different temperatures. The data obtained have been placed in table 3. From the table it is apparent that mycelial elaboration was maximum at 28^0C and minimum at 20^0C. It may be noted that either decreasing or increasing range of temperature had negative impact.

Similarly aflatoxins elaboration was also the maximum at 28^0C. Further B_1 & G_1 were elaborated more quantitatively i.e. 3.8 mg /100, and 2.8 mg/100 ml of culture filtrate respectively. The elaboration of aflatoxins were affected by both the decreasing or inscreasing temperature. Both had negative impact.

Six different carbon sources at same concentration were used separately and mycelial growth and aflatoxins were noted and data have been presented in table 4. From the table it is clear that out of the six different carbon sources viz., Ribose, Fructose, Glucose, Sucrose, Raffinose and Mannitol, Glucose promoted that maximum mycelial growth i.e., 2.8g / 100 ml of culture. This was followed by sucrose. This was also true for aflatoxin elaboration which was 3.8 mg / 100 ml of culture in glucose containing medium while 3.7 mg in sucrose. It may be further noted that aflatoxin G_1 was produce next to B_1 which was 3.4 mg / 100 ml of culture in glucose and 2.60 in sucrose. Here aflatoxin B_2 and G_2 were also produced that was 0.7 mg / 100 ml and 0.40 mg / 100 ml of culture medium respectively. On sucrose this amount was 0.52 mg / 100 ml and 0.30 mg 100 ml of culture.

On mannitol non of the aflatoxins were produced, where as on ribose only B_1 and G_1 were produced.

pH influences activities of the enzymes. This may be correlated with the above findings that enzyme involved in the synthesis of secondary metabolites may be influenced at very low and high pH, resulting in reduced synthesis of aflatoxins. So we got lesser amount at these pH. 5.5 pH may be congenient for better activities of these enzymes. Similar assumption may be true for different temperatures also. Here neither lower nor the higher temperatue could promote maximum mycelial growth nor aflatoxin elaboration. Here also enzymes may be affected by such temperature range.

Different sources of carbon and their concentrations significantly influence the growth of fungi and also their capabilities to produce aflatoxins. Danis et al; (1966), Hesseltins et al; (1966), Shih and Marth (1975), Doyle and Marth (1978a), and Doyle and Marth (1978b) have reported the impact of initial pH on mycelial growth and aflatoxin elaborations. They reported suitable pH may range between 4.5 to 5.4 for mycelial growth and aflatoxin production. Present finding also corroborate with the above.

Hesseltime (1966) and Sorenson et al; (1967) noted that 28^{0+C} is the most congenient temperature for mycelial growth and aflatoxin elaboration in *A. parasiticus*. Therefore, present finding is supported by the above reports.

Effects of different carbon sources on mycelial growth and aflatoxin elaboration by different toxigenic strain have been reported by Hesseltine (1966 and 1970); Diener et al(1966); Davis and Diener (1968); Shih and Marth (1974), Prasad 1983. In the present study out of the six carbon sources used here, glucose was found most suitable carbon source for mycelial yield and elaboration of aflatoxins. All the four aflatoxins were elaborated here but B_1 (3.82 mg / 100 ml culture filtrate) was the highest which was followed by G_1 i.e., 3.40 mg. Similarly B_2 was elaborated at higher concentration than G_2. Sucrose was next to glucose. Above finding is accordance with the findings of Matelis and Adye (1965), Davis et al (1966); and Shih and Marth (1974), who reported glucose as excellent sources of carbon. Therefore, the present result corroborate their findings as well.

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Table -1

Showing Observed and Expected R f values of the Aflatoxins.

Spots	Observed R f value	Expected R f value	Differences
B1	0.558	0.56	0.036
B2	0.529	0.53	0.019
G1	0.475	0.48	0.105
G2	0.458	0.46	0.044

Table-2

Effect of Initial pH on mycelial Growth and aflatoxin production by *A. parasiticus* strain 3000

Initial pH	Dry weight of mycelial Mat g/100ml of medium	Qualitative detection of Aflatoxins				Quantitative estimation of aflatoxins mg/100ml of culture filtrate
		B1	B2	G1	G2	B1 B2 G1 G2
3.5	2.364	+	-	+	-	2.560- 2.250
4.5	3.246	+	T	+	T	3.250- 2.360
5.5	3.775	+	T	+	T	3.750- 2.850
6.5	2.840	+	T	+	T	2.654- 2.350
7.5	1.016	+	-	+	-	1.650- 1.300

+(PRESENT), - (ABSENT), T (TRACE)

TABLE-3

Mycelial Growth of and Aflatoxin Elaboration by *A. parasiticus* strain 3000 at different temperature

Temperature in 0^C	Dry weight of Mycelial mat g/100 ml of culture medium	Qualitative detection of Aflatoxins				Qualititative estimation of Aflatoxins mg/100 ml of culture filtrate			
		B1	B2	G1	G2	B1	B2	G1	G2
20	1.680	+	-	+	-	0.560 1.880 3.750	- - -	0.50 1.620 2.820	- - -
24	2.530	+	-	+	-	2.850	-	2.540	-
28	3.870	+	T	+	T	2.080 -	- -	1.864 -	- -
32	3.580	+	T	+	T				
36	3.428	+	T	+	T				
40	2.450	T	-	+	-				

+ (Present),
- (Absent),
T (Trace)

Table - 4

Mycelial growth of and Aflatoxin Elaboration by *A. parasiticus* Strain 3000 on different carbon

Carbon sources	Dry weight of mycelial mat g/100ml of medium	Qualitative detection of Aflatoxins.				Quantitative estimation of Af;atpxoms mg/100 ml of culture filtrate.			
		B1	B2	G1	G2	B1	B2	G1	G2
Ribose	2.38	+	-	+	-	2.68	-	1.72	-
Fructose	2.46	+	+	+	+	3.48	0.68	3.25	0.34
Glucose	2.80	+	+	+	+	3.82	0.74	3.40	0.40
Sucrose	2.72	+	+	+	+	3.70	0.62	2.96	0.36
Raffinose	2.56	+	+	+	+	3.40	0.56	2.60	0.25
Mannitols	2.28	+	-	+	-	-	-	-	-

+ (PRESENT)
- (ABSENT)

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