Factors Affecting Aflatoxin Production by *Aspergillus parasiticus* in a Chemically Defined Medium

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The optimum levels of sucrose, $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 , KH_2PO_4 and ZnSO_4 for aflatoxin production in a chemically defined medium have been established. The last two were found to be essential for fungal growth and aflatoxin production. The effect of various carbon sources on aflatoxin production was tested using the defined medium. Asparagine was found to be essential for aflatoxin production. Very little aflatoxin was produced in the absence of asparagine with any of the other inorganic nitrogen sources tested. Supplementation with yeast extract, Casamino acids, Casitone and peptone increased the aflatoxin yield, but omission of asparagine led to decreased aflatoxin yields even when complex nitrogen sources were present. Asparagine could be replaced by aspartic acid or alanine.

INTRODUCTION

The effect of various carbon and nitrogen sources on aflatoxin production in liquid cultures has been studied by a number of workers (Eldridge, 1964, 1965; Mateles & Adye, 1965; de Iongh *et al.*, 1965; Davis *et al.*, 1967; Davis & Diener, 1968; Diener & Davis, 1969; Naik *et al.*, 1970). The ability to utilize inorganic or organic nitrogen and carbon sources for toxin formation depends on the strain of fungus used, the composition of the basal medium, pH and temperature.

A synthetic medium (SL medium) which supports growth and high toxin yields has been described previously (Reddy *et al.*, 1971). In this investigation, the effect of various simple and complex nitrogen and carbon sources on aflatoxin formation was studied making use of SL medium.

METHODS

Aspergillus parasiticus ATCC 15517 (Northern Regional Research Laboratory, Peoria, Ill., U.S.A.) was maintained as a soil culture. Spores (5 d old) harvested from a bottle containing 50 ml of glucose/peptone agar were distributed equally between five 500 ml Erlenmeyer flasks each containing 100 ml of sterile SL medium (Reddy *et al.*, 1971). In the experimental series, sucrose was replaced by different carbon sources keeping the carbon content of the medium constant. Various nitrogen sources were included in the medium both as additives and as substitutes for $(\text{NH}_4)_2\text{SO}_4$. Substitution of $(\text{NH}_4)_2\text{SO}_4$ by inorganic nitrogen sources was done on an equal nitrogen basis. Flasks were incubated at $26 \pm 1^\circ\text{C}$ as stationary cultures for 8 d. Experiments were carried out in duplicate and average results are reported. Individual variation between flasks was always less than 10%.

The media and mycelia were separated and wet and dry weights of the mycelium were determined. Aflatoxins were extracted with chloroform, separated by thin-layer chromatography on silica gel G in toluene/isoamyl alcohol/methanol (90:32:3, by vol.) (Reddy *et al.*, 1970), eluted with methanol and estimated by measuring the absorption at 363 nm as described by Nabney & Nesbitt (1965).

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Table 1. *Optimum concentrations of various constituents of SL medium or aflatoxin production by A. parasiticus ATCC 15517*

Constituent	Concn (g l ⁻¹)	Mycelial dry wt [g (100 ml medium) ⁻¹]	Aflatoxins [mg (100 ml medium) ⁻¹]				Total
			Medium		Mycelium		
			B	G	B	G	
Sucrose	80	2.98	2.31	9.73	3.59	13.02	28.65
(NH ₄) ₂ SO ₄	3.5	3.24	3.39	13.02	3.51	10.53	30.45
KH ₂ PO ₄	0.75	2.70	1.52	12.45	3.00	10.90	27.87
MgSO ₄ ·7H ₂ O	0.5	3.93	2.13	11.60	4.60	12.85	31.18
ZnSO ₄ ·7H ₂ O	0.025	3.34	3.88	9.92	4.20	9.87	27.87

RESULTS AND DISCUSSION

The optimum concentrations of sucrose, (NH₄)₂SO₄, KH₂PO₄, MgSO₄ and ZnSO₄ for aflatoxin production are presented in Table 1. Magnesium was found to be essential for aflatoxin formation and fungal growth.

The effects of substitution of sucrose by other carbon sources are shown in Table 2. Glucose supported good fungal growth and toxin yield. Maltose was better than glucose and sucrose in promoting fungal growth, but the total aflatoxin yields were lower with maltose and the proportion of aflatoxin B was higher than that obtained with sucrose and glucose. With arabinose, reasonable yields of aflatoxin B were obtained but very low yields of aflatoxin G. Starch and fructose were poor substrates for aflatoxin production.

Mateles & Adye (1965) and Davis *et al.* (1967) found that glucose and sucrose were equally efficient in supporting aflatoxin formation. Bennett *et al.* (1976) reported that higher concentrations of glucose in resting cell medium gave higher yields of aflatoxins. Reports on the effect of fructose differ. Mateles & Adye (1965) reported good aflatoxin yields whereas Davis *et al.* (1967) obtained low yields, but found a mixture of fructose and glucose to be comparable with glucose. In this study, when the sucrose in SL medium was replaced by either fructose or a mixture of glucose and fructose (1:1), aflatoxin yields were markedly reduced (Table 2).

Glycerol gave good yields on SL medium (Table 2). When ethanol was substituted for sucrose there was less growth and toxin formation [about 10 mg aflatoxin (100 ml medium)⁻¹], and the concentration of aflatoxin G in the medium was greater than in the mycelium. Basappa *et al.* (1967) reported that 2% ethanol increased toxin production nearly fivefold compared with the control. In our study there was no growth with either acetic acid or oxalic acid. All single amino acids tested as sole carbon source failed to support aflatoxin formation.

Ammonium acetate, NH₄Cl and NaNO₃ were found to be comparable to (NH₄)₂SO₄ as nitrogen sources whereas urea and NH₄NO₃ did not favour aflatoxin production (Table 3*a*). Omission of asparagine from the medium could not be compensated for by any of the inorganic nitrogen sources tested (Table 3*b*). Supplementation of SL medium with complex nitrogen sources resulted in enhanced aflatoxin formation (Table 3*c*). Addition of yeast extract gave maximum growth and aflatoxin production. Under these conditions, omission of (NH₄)₂SO₄ from the medium had no effect on growth and toxin yield. Davis *et al.* (1967) reported that with their medium, which contained glycine and KNO₃ as nitrogen sources, omission of glycine drastically reduced the aflatoxin yield and omission of KNO₃ reduced yields from 2.38 to 1.18 mg (100 ml medium)⁻¹.

The effect of complex nitrogen sources on aflatoxin production also varies with the strain and medium used. Peptone and yeast extract were reported to stimulate toxin production by *A. flavus* (Davis *et al.*, 1967; Maggon *et al.*, 1969), but not by *A. parasiticus* ATCC 15517 (Mateles & Adye, 1965). Casamino acids stimulate aflatoxin formation (Mateles & Adye,

Table 2. *Effect of different carbon sources on aflatoxin production by A. parasiticus ATCC 15517 in SL medium*

Carbon source	Mycelial dry wt [g 100 ml medium) ⁻¹]	Aflatoxins [mg (100 ml medium) ⁻¹]				
		Medium		Mycelium		Total
		B	G	B	G	
Sucrose	3.09	1.81	10.10	4.57	11.36	27.84
Glucose	3.42	1.69	12.56	4.98	7.01	26.24
Maltose	4.85	2.12	4.69	6.12	8.60	21.53
Fructose	2.32	0.24	2.75	1.26	3.42	7.67
Glucose+fructose (1:1)	2.63	0.52	3.95	1.72	5.79	11.98
Arabinose	3.04	0.28	1.42	1.88	5.72	9.30
Starch*	2.95	0.25	1.26	1.51	1.44	4.46
Citric acid	1.04	0.14	0.54	0.28	0.55	1.51
Aspartic acid	0.18	0.05	0.04	0.05	0.06	0.20
Ethanol	1.60	1.13	5.15	1.23	2.92	10.43
Glycerol	3.27	1.25	10.00	4.40	8.80	24.45

* Did not dissolve completely at the concentration used.

Table 3. *Effect of different nitrogen sources on aflatoxin production by A. parasiticus ATCC 15517*

In (a), the added nitrogen source replaced only the $(\text{NH}_4)_2\text{SO}_4$ present in SL medium; asparagine was present in this medium. The total nitrogen content of all media was 2.85 g l^{-1} . In (b), the medium used did not contain asparagine. Again, the nitrogen content was maintained as 2.85 g l^{-1} . In (c), SL medium was supplemented with nitrogen sources at 2% (w/v).

Nitrogen source	Mycelial dry wt [g (100 ml medium) ⁻¹]	Aflatoxins [mg (100 ml medium) ⁻¹]				Total
		Medium		Mycelium		
		B	G	B	G	
(a) Nitrogen source added to (NH ₄) ₂ SO ₄ -deficient SL medium						
None	2.48	1.50	6.37	2.24	2.92	13.03
Urea	2.12	2.61	5.82	2.58	2.13	13.14
NH ₄ NO ₃	2.65	2.31	5.45	2.12	2.14	12.02
NaNO ₃	2.52	4.82	8.17	5.95	5.57	24.51
(b) Nitrogen source added to asparagine-deficient SL medium						
None	0	0	0	0	0	0
Urea	0.15	0.50	0.12	0.47	0.17	1.26
NH ₄ NO ₃	1.18	0.64	0.33	0.52	0.32	1.81
NH ₄ acetate	1.68	1.01	0.47	0.89	0.46	2.83
(NH ₄) ₂ SO ₄	0.69	0.88	0.32	0.56	0.29	2.05
NH ₄ Cl	1.52	0.87	0.41	0.74	0.35	2.37
NaNO ₃	1.61	0.94	0.38	0.69	0.28	2.29
(c) Nitrogen source added to SL medium						
None	3.18	3.19	11.50	4.20	9.86	28.75
Casamino acids	4.80	1.65	11.19	7.94	18.80	39.58
Casitone	3.27	5.28	10.27	14.52	12.21	42.28
Peptone	4.27	1.51	6.60	9.14	22.98	40.23
Yeast extract	5.98	2.89	13.80	10.24	23.28	50.21

1965). These substances can be expected to provide asparagine, aspartic acid or alanine which are known to be essential for high aflatoxin production. In this investigation it was found that even in the presence of these complex nitrogen sources, omission of both $(\text{NH}_4)_2\text{SO}_4$ and asparagine from the SL medium reduced the yield of aflatoxin by 50%; the reduced yield was 30% less than that obtained on SL medium alone. Complex nitrogen

Table 4. *Effect of different amino acids on aflatoxin production by A. parasiticus* ATCC 15517

Asparagine in SL medium was replaced by other amino acids on an equal nitrogen basis. $(\text{NH}_4)_2\text{SO}_4$ was present as an inorganic nitrogen source.

Amino acid	Mycelial dry wt [g (100 ml medium) ⁻¹]	Aflatoxins [mg (100 ml medium) ⁻¹]				
		Medium		Mycelium		Total
		B	G	B	G	
None	0.75	0.74	0.28	0.59	0.25	1.86
L-Asparagine	3.07	3.87	10.07	4.02	10.19	28.15
DL-Alanine	4.40	2.80	8.83	6.19	9.84	27.66
DL-Aspartic acid	3.64	4.25	5.71	9.60	6.90	26.46
Glycine	3.17	1.28	7.57	1.91	7.20	17.96
L-Glutamic acid	2.08	2.87	2.10	3.70	1.40	10.07
L-Methionine	1.28	2.11	1.45	1.24	0.40	5.20
DL-Leucine	1.56	1.78	0.70	1.94	0.17	4.59
L-Arginine.HCl	1.21	1.40	0.07	0.60	0.13	2.20
L-Lysine.HCl	1.26	0.98	0.40	0.21	0.06	1.65
DL-Phenylalanine	1.51	1.60	0.71	0.77	0.05	3.13
DL-Tryptophan	1.86	1.47	0.60	1.68	0.15	3.90
L-Tyrosine.HCl*	0.78	0.73	0.15	0.25	0.29	1.42

* Did not dissolve at concentration used.

sources were able to substitute completely for $(\text{NH}_4)_2\text{SO}_4$, since omission of $(\text{NH}_4)_2\text{SO}_4$ did not affect aflatoxin yields.

Yeast extract was particularly efficient in promoting toxin production, since maximum yields were obtained on SL medium containing 2% (w/v) yeast extract (Table 3c). It was also more efficient than the other complex nitrogen sources in replacing asparagine since the yield of the toxin was reduced by only 20%. The best medium available before the development of SL medium was a semi-synthetic medium containing sucrose and yeast extract (Davis *et al.*, 1967; Maggon *et al.*, 1969).

The effect of amino acids on aflatoxin formation also depends on the strain and the growth conditions employed. Earlier workers have used amino acids as sole nitrogen sources. They reported that glycine and glutamic acid could replace $(\text{NH}_4)_2\text{SO}_4$ with respect to growth and aflatoxin formation, whereas alanine failed to promote good growth with aspartic acid, glutamic acid and glycine which, however, gave only 50% of the toxin yields obtained with the control. Alanine and asparagine also supported good growth but gave very low yields of aflatoxins (Davis *et al.*, 1967; Mateles & Adye, 1965). In asparagine-deficient SL medium, although small amounts of aflatoxins B_1 , B_2 and G_2 were present, no aflatoxin G_1 was formed (Table 4). Aspartic acid and alanine were found to be comparable to asparagine in their ability to support aflatoxin formation (Table 4). Glycine and glutamic acid were less effective. These amino acids also supported better growth than did SL medium containing $(\text{NH}_4)_2\text{SO}_4$ as sole nitrogen source. The effect of these amino acids was not due to the extra nitrogen provided, since replacement of asparagine by $(\text{NH}_4)_2\text{SO}_4$ on an equal nitrogen basis decreased the yield of aflatoxin. Asparagine, aspartic acid and alanine can be expected to be metabolized to pyruvate and hence acetyl coenzyme A. It is possible that these amino acids provide extra amounts of metabolites which can be utilized in the synthesis of both primary and secondary metabolites. This may, in part, explain the enhanced growth and aflatoxin yields.

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