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INFLUENCE OF *IN VITRO* FACTORS ON THE PRODUCTION OF OCHRATOXIN A BY *ASPERGILLUS OCHRACEUS*

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ABSTRACT

Ochratxoxin A (OTA) produced by some species of Aspergillus and Penicillium is a highly potent toxin among all mycotoxins. OTA biosynthesis by fungi is influenced by many intrinsic and extrinisic factors. The present paper presents the results pertaining to studies on the effect of in vitro factors (different media, incubation period, pH and temperature) on the production of OTA by Aspergillus ochraceus. These studies revealed that A.ochraceus is able to grow on a wide range of substrates. Of the 15 types of substrates employed, yeast extracts sucrose (YES) +5% bee pollen medium supported production of large amounts of OTA despite the optimal production of biomass. Under cultural conditions, production of OTA began from 9th day and reached peak level by 21st day. There onwards a gradual decrease was observed. This may be due to the fact that the strains sequester and assimilate the phenylalanine moiety of OTA molecule as other nitrogen sources in the culture medium become exhausted. OTA production was also influenced by change of the pH of culture medium. A temperature of 30^{0} C was found to be optimum. The ability to produce OTA over a wide range of temperatures, enable the organism to produce the toxin during storage at different climatic conditions.

KEY WORDS

Ochraxin A, Aspergillus ochraceus, medium, pH, temperature, incubation period.

INTRODUCTION

Ochratoxins produced by species of *Aspergillus* and *Penicillium* gained prominance in the last decade because of their widespread occurrence on different foods and feeds, and their toxic potentiality in humans and livestock. Ochratoxins are the first major group of toxins discovered after aflatoxins [1]. Among three types of ochratoxins viz., Ochratoxin A, B, C, Ochratxoin A is highly toxic and more prevalent than the other two compounds. OTA is a widespread mycotoxin produced by several *Penicillium* and *Aspergillus* species, mainly by *P. verrucosum* and *P. nordicum* [2,3] *A. ochraceus* and *A. carbonarius* [4-7] together with a low percentage of closely related species of *Aspergillus* such as *A. niger* [8,9] and *A. tubingensis* [10,11]. Our studies conducted on the incidence of mycotoxigenic fungi and mycotoxins in poultry feeds revealed that many samples were extensively contaminated by ochratoxin A produced by *A. ochraceus*.

In *Aspergillus* species, OTA synthesis depends on several environmental factors [12]. Some authors have reported the effects of culture conditions on OTA production by *Aspergillus* strains. The results obtained were as diverse as the conditions applied. Changes in pH, composition of culture media, trace metals, and carbon and nitrogen sources may have a remarkable influence [13, 14]. In the present investigations, an attempt was made to assess the different physical factors (culture media, incubation period, temperature and pH) on the production of OTA.

MATERIALS AND METHODS

Organism, source and identification

The toxigenic *Aspergillus ochraceus* strains used in the present investigations were isolated from poultry feeds

of Warangal district. The identification was confirmed by Dr. P. N. Chowdhry, Principal Mycologist, National Centre of Fungal Taxonomy, New Delhi with accession no: 5182.12

Qualitative and quantitative analysis of ochratoxin A

Isolates of *A. ochraceus* were grown in yeast extract sucrose (YES) medium for 20 days at 27 ± 2^{0}C. At the end of incubation period, cultures were harvested and the filtrate was acidified by adding 1 N HCl and extracted twice with the equal amount of chloroform and evaporated to dryness. The extract was spotted on TLC plates and run in toluene: ethyl acetate: formic acid (6:3:1 v/v) solvent system. The spots developed by spraying with NaOH or ammonia were observed under UV light. The ochratoxin A appeared as blue fluorescent spot [15, 16,]. The quantitative estimation of OTA was done by spectrophotometric method [17].

Confirmation of OTA through liquid chromatography tandem mass spectrometric method

Mass spectrometry was performed on triple quadrupole LC/MS/MS mass spectrometer (AB Sciex Instruments, Singapore, API 4000), a method suggested by [18] with the kind courtesy of Indian Institute of Toxicology Research (IITR), Lucknow.

Studies on *Aspergillus ochraceus* with reference to ochratoxin A production

Detailed investigations were undertaken on production of OTA by *A. ochraceus* with some experimental modifications. *A. ochraceus* was grown in 25ml of different media (Table 1) for 20 days. At the end of the incubation period, pH of the culture filtrate was determined with the help of Hanna digital pH meter. Biomass attained by *A. ochraceus* was determined by dry weight method. At the end of 20 days of incubation period the mycelium was harvested on Whatman No. 1 filter paper. The harvested mycelia were washed several times with water. The filter paper along with the fungal mycelium was squeezed and dried at 65- 70^{0}C for 48 hrs. The dried filter paper was weighed to a constant weight after bringing to room temperature by keeping in desiccators.

RESULTS AND DISCUSSION

1. Influence of culture media

A. ochraceus was grown in different synthetic media and the mycelial growth and OTA production was recorded and the results are summarized in Table 1.

Table 1: Influence of different culture media on biomass and OTA production by *A. ochraceus*

Culture media	Final pH	Dry weight (mg/ml)	OTA (µg/ml)
Aflatoxin production medium	7.23	2.10	2.19
Asthana & Hawkers medium	5.25	7.30	2.17
Coconut broth medium	3.10	12.05	2.81
Czapek yeast extract medium	2.15	6.05	5.31
Maize flour medium	4.15	0.90	6.85
Malt extract medium	4.19	12.00	2.98
Nutrient broth medium	6.23	1.20	1.19
Potato dextrose broth medium	5.10	19.65	8.01
Richards medium	2.10	9.80	19.68
Rice flour medium	5.65	21.05	7.10
Wheat flour medium	2.13	18.05	9.12
Wickerham medium	7.10	8.90	12.08
Yeast extract sucrose medium	3.10	26.35	28.95
Yeast extract sucrose medium+5% bee pollen	3.15	9.05	30.21

Initial pH was adjusted to 6.5

ANOVA

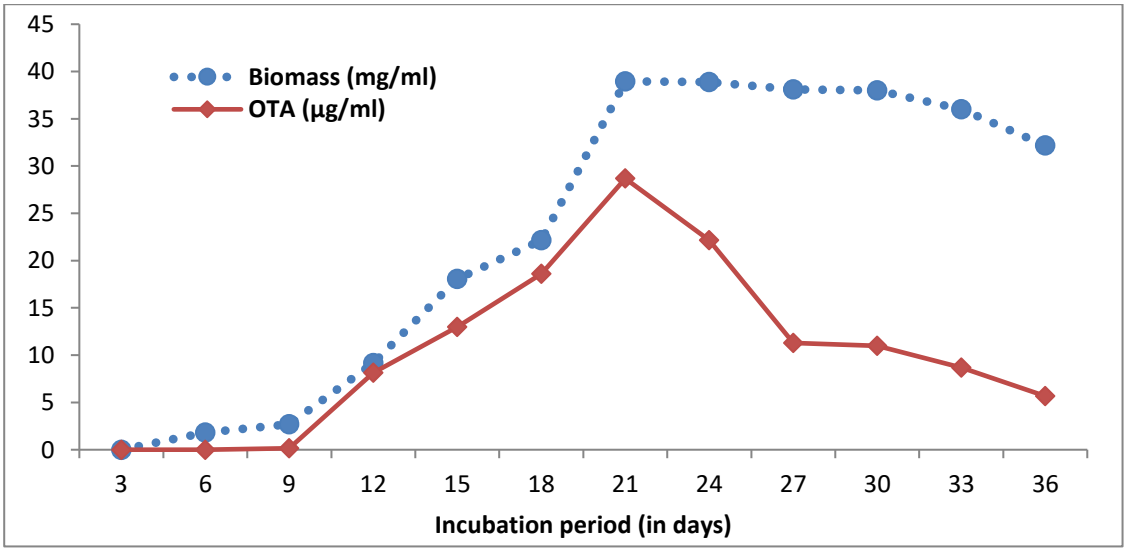
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2334.7547	13	179.5965	359.193	p<0.001	2.5073
Within Groups	7	14	0.5			
Total	2341.7547	27				

From the critical study of the Table 1, it is evident that *A. ochraceus* produced maximum amount of OTA on yeast extract sucrose+5% bee pollen and next in YES, Richards medium. Wickerham medium, potato dextrose broth medium and the food based (flour) mediums maize flour medium, rice flour medium, wheat flour medium supported intermediate production of OTA by *A. ochraceus*. Rest of the media supported the least amount OTA production. The maximum growth of *A. ochraceus* was recorded in YES medium followed by food based (flour) mediums wheat flour medium, rice flour medium and in potato dextrose broth medium. YES+5% bee pollen, Richards medium, MEA, CBM mediums supported fairly good fungal growth. Rest of the mediums did not favor the fungal biomass production. Interestingly, food based (flour) maize flour medium did not support the *A. ochraceus* growth. The final pH of most of the mediums was acidic in nature, except aflatoxin production medium, Wickerham medium which showed alkaline in final pH but mycelium growth achieved was entirely different. From the above findings indicates that there is no correlation between the pH and mycelial growth and OTA production by *A. ochraceous.* But a similar final pH was recorded in YES medium and YES + 5% bee pollen medium, which are responsible highest production of OTA by *A. ochraceus*. It can be concluded from the above results that pH plays a limited role on OTA production by *A. ochraceus*. The results obtained in these studies are in agreement with the observations made by [13] who reported bee pollen can be regarded as a natural medium for fungal growth much superior than many cereals [19]. However, in a recent study carried out with *A. ochraceus* strains isolated from different substrates, a lack of substrate specificity for OTA production was noticed [20]. Moreover, the ochratoxigenic ability of *A. ochraceus* was independent of the culture substrate, and OTA production by the same fungal isolates in various substrates was quite different even under the same environmental conditions.

2. Influence of different incubation periods

This study was undertaken to determine the effects of different incubation durations on total OTA production by *A. ochraceus* and the results are presented in the Figure 1.

Figure 1: Influence of different incubation periods on biomass and OTA production by *A. ochraceus*



From the figure 1 it is evident that OTA production by *A. ochraceus* started only after a certain lag period i.e., after 6 days. It is also evident from the table that OTA production increased with the progress of incubation period and reached optimum production at 21st day. The amount of OTA decreased marginally with the progress of incubation period. Similar results were obtained by [21,22,23].

3. Influence of different p^{H}

Fungal growth is strongly affected by pH [24]. Influence of pH on different *Aspergillus* species was studied by [24, 25]. However, limited information is available on the production of OTA by *A. ochraceus* at different pH

ranges. Hence, in the present studies effect of pH on growth and OTA production by *A.ochraceus* was investigated.

Table 2 : Infuence of different pH on biomass and OTA production by *A. ochraceus*

Initial pH	Final pH	Dry weight (mg/ml)	OTA (µg/ml)
3.5	4.28	8.56	ND
4.5	4.80	13.58	3.28
5.5	4.90	22.10	12.18
6.5	4.28	28.10	18.28
7.5	4.36	11.87	16.26
8.5	4.18	6.86	12.16
9.5	4.98	4.83	ND
10.5	5.10	3.32	ND

ND = Not detected

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	830.688775	7	118.669825	464.2340342	p<0.001	3.5005
Within Groups	2.045	8	0.255625			
Total	832.733775	15				

From the Table 2 it is evident that maximum amount of OTA was produced at pH 6.5. The production of OTA was observed in the pH range of 4.5 to 8.5. However, no production of OTA was found beyond pH 9.5. The final pH recorded was completely acidic. A correlation could be drawn between the pH of the medium and fungal biomass. The fungal biomass steadily increased from pH 3.5 to 6.5. The results are in agreement with the results of [14], who found that OTA production depends on the initial pH and was influenced by change of the pH of culture medium.

4. Influence of different temperatures

The most significant factor capable influencing the OTA production by fungi is temperature [26]. The impact of temperature on OTA contamination was studied by several authors. A study conducted by [23] on several strains of *A.niger* revealed that *A. niger* strains achieved maximum OTA levels in YES medium predominantly at 20-25^{o}C, while *A. carbonarius* strains produced the highest OTA levels in CYA medium at 15-20^{o}C. Clouvel *et al.* [27] and Oueslati *et al.* [28] also studied the effect of temperature on different species of *Aspergillus*. However, limited information is available on the effect of different temperatures on OTA production by *A.ochraceus*. Hence, in the present studies, effect of different temperatures on growth and OTA production by *A.ochraceus* was investigated and the results are summarized in Table 3.

From the table 3 it is evident that there is a significant influence of temperature on growth and OTA production by *A.ochraceus*. Maximum amount of OTA was produced by *A. ochraceus* at the temperature 30^{o}C. Above this temperature there was a sudden decrease in the amount of OTA production by *A.ochraceus*. OTA production and mycelial growth were completely inhibited at incubation temperature of 10^{o}C. These results are slightly in contrast with the findings of Belli *et al.* [29] who reported that the optimum temperature range for OTA production was at 15-20^{o}C.

CONCLUSIONS

the present investigations reveal that YES+bee plollen medium, incubation period of 12 days, pH 3.5 to 6.5 and 30^{0}C temperature are ideal for the production of ochratoxin A by *A. ochraceus.*

Table 3: Influence of different temperatures on biomass and OTAproduction by *A. ochraceus*

Temperature in °C	Final pH	Dry weight (mg/ml)	OTA (µg/ml)
10	6.85	NG	ND
15	5.85	3.15	3.16
20	6.90	13.15	8.17
25	5.30	20.38	13.15
30	6.92	28.16	26.15
35	5.03	27.12	9.12
40	6.30	18.16	2.12

Initial pH was adjusted to 6.5 NG = No growth, ND = Not detected

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1414.394724	6	235.732454	3076.29973	p<0.001	2.85
Within Groups	1.0728	14	0.076628571			
Total	1415.467524	20				

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