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Comparative Quantification by HPLC-FLD of Ochratoxin production by *Aspergillus ochraceous* MTCC 4643

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ABSTRACT

Ochratoxin is a mycotoxin produced by *Penicillium* and *Aspergillus* species in different food grains, when it it is kept in hot and humid environmental conditions. Ochratoxins are known to be carcinogenic, mutagenic, teratogenic and immunosuppressive in nature. Ochratoxins occur more frequently in cold environment condition but some strain of *A. niger* and *A. ochraceous* were able to grow in tropical and subtropical climates, which is same as of India. The deterioration of stored food grain with *A. niger* and *A. ochraceous* reported in higher amount as compared to other ochratoxicogenic fungal species. This form of contamination has a direct impact on human staple foods and is linked to illness caused by ochratoxin contamination. Because ochratoxin has a high tolerance limit in humans, it is not easily recognized and causes long-term problems such as cancer associated with liver and kidney. The present study focuses on the production of ochratoxin on known MTCC toxicogenic strain of MTCC 4643 *A. ochraceous* in different semisynthetic and natural media. The concentration of ochratoxin by HPLC-FLD is found to be greater in coffee medium with 35% moisture content than in rice medium with 35% moisture content and SKMY medium. As a result, the coffee medium may be employed as a suitable natural substrate for the generation of ochratoxin in greater quantities than other current synthetic media. Also it is found to be cost effective as compared to synthetic or semisynthetic medium.

Key words: *A. ochraceous, Ochratoxin A, HPLC-FLD, Aspergillus species, Rice medium, Coffee medium, SKMY medium*

Introduction

Mycotoxins are toxic chemical substances and secondary metabolites generated by fungi and moulds. Food-borne mycotoxins have immediate impacts, resulting in severe disease symptoms (Abarca, 2000). Mycotoxins that do occur in food and/or feedstuffs have a significant impact on human and cattle health. Mycotoxins are connected with damaged or mouldy crops since they are generated by fungus, albeit apparent mould contamination might be superficial (Bennett, 1987). Some *Aspergillus* and *Penicillium* species are the primary producers of OTA. *Aspergillus* and its teleomorph, Eurotium is the pinnacle of spoiling fungus. Several mycotoxins are produced by the genus *Aspergillus* and its members, which have a significant agricultural, epidemiological, and economic impact (Pitt and Hocking, 1999). It is one of the primary agents responsible for mycotoxin agricultural pollution, and it is linked to the

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common microbial flora of many agronomic crops. When the spores discover the required nutrients and favorable environmental circumstances, the fungus colonizes swiftly and produces a variety of toxins.Primarily Ochratoxin A was discovered as a metabolite of *Aspergillus ochraceus* later, ochratoxinA production is mostly related with *A. carbonarius* and *A. niger* or nigris section species (Futagami *et al.,* 2011).*A. niger* is one of the most prevalent species of this genus, with a wide worldwide range, among the fungus most usually recorded from foods (Palumbo *et al.,* 2008). Aspergillus has a wide spectrum of growth. It was discovered that the growth temperature and pH ranges are between 10-50 °C and 2.0-11.0, respectively, and that it is osmotolerant at salt concentrations of up to 34% (Lubertozzi *et al*., 2009). Intrinsic factors (water activity, plant attributes, the nature of these substrates, and nutrient composition), extrinsic (climate change), and implicit (type of microorganism, spore load or concentration in food, and microbiological ecosystem) are among the conditions that favors fungal infection and mycotoxin production in preharvest foods (Magan *et al.,* 2007). Processing elements, such as agronomic practices, are added to this. Some of these rotting conditions begin before harvest, whereas others begin after harvest (Jay *et al*., 2005).

This species is more common in warmer areas, both in the wild and in preserved foods. The black spores appear to produce circumstances that protect against sunlight and UV rays, giving them a competitive edge in such environments (Hayat *et al*., 2012). Only ochratoxin is possibly as significant as the aflatoxins among Aspergillus toxins (Beardall *et al.,* 1994). Kidney is the primary target organ of this toxic; it is potent nephrotoxic in nature (Kuiper-Goodman *et al.,* 1989). In addition to being a nephrotoxic in nature, animal studies also show that the ochratoxin A is a liver toxin, an immunological suppressant, a powerful teratogen, and a carcinogen. Despite the fact that OTA is a significant mycotoxin for food safety and human health, the genetic basis of OTA biosynthesis is little understood in comparison to other major mycotoxins such as aflatoxins, fumonisins, and zearalenone (Kuiper-Goodman *et al*., 1989). OTA has a substantial economic impact on food commodities since OTA generating fungi have been discovered to be a contaminant in a wide range of foodstuffs (Pitt and Hocking, 2009). When the structure of OTA is examined, it is discovered to be a polyketide-derived secondary metabolite with a dihydrocoumarin moiety connected by an amide bond to an L—phenylalanine (Phe) obtained from the shikimic acid pathway. L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1H-2-benzopyrane-7-yl) carbonyl] is its chemical name.-(R)-isocoumarinL-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1H-2-benzopyrane-7-yl) carbonyl] is its chemical name.-(R)-isocoumarin (Khoury *et al*., 2010). OTA has been linked to urinary tract tumours and has been linked to the aetiology of Balkan endemic nephropathy (BEN) (Kuiper-Goodman *et al*., 1989). The present investigation focuses on the production of ochratoxin A from a standard toxicogenic strain of *A. Ochraceous* in different medium and comparative yield analysis with the help of HPLC-FLD.

Materials and Methods

Chemical and reagents

All the media ingredients of CYA(Czapek's Yeast Extract Agar), YES (150 g sucrose, 20 g yeast extract and 1000 mL of distilled water) were purchased from sigma-aldrich. The entire chemical reagents bought are of HPLC grade. Ochratoxin standard were purchased from R-Biopharm Neugen Pvt. Ltd., Hyderabad, India, in a concentration of 10 µg/ml in methanol. The standard was stored at 4 °C away from light before use.

Source of toxicogenic fungal strain

Ochratoxin producing strain of *Aspergillus ochraceous* MTCC 4643 obtained from Microbial Type Culture Collection, Chandigarh, India. The fungal strain was propagated on CYA slants and the morphological and microscopic characters of the culture were studied. Pure culture was maintained at 4 °C until use in the form of slant in triplicates.

Detection and production of ochratoxin in YES culture condition

The spores of *A. ochraceous* MTCC 4643 were aseptically taken with 1µl inoculatingnichrome loop and transferred into broth medium in triplicates of YES medium and incubated under the static condition at room temperature for 15 days. In YES medium mold broth filtered with normal filter paper followed by Whatman No.1 filter paper and the filtrate was collected in the flask. In this filtrate equal volume of chloroform was added and kept in shaking condi-

tion for 30 minutes. The chloroform fraction was collected through separating funnel and then it is passed through sodium sulphate column to remove the moisture content of the fraction. This fraction were subjected to thin layer chromatography containing TLC Silica gel 60 F_{254} plate 20 cm X 20 cm (Merck, Dermstadt, Germany) as stationary phase and a mixture of Toluene: Ethyl acetate: 90% Formic acid (5:4:1,v/v) as the mobile phase. Ochratoxin A was confirmed by the presence of fluorescence band having Rf value same as of standard visualized under UV light using a UV-transilluminator.

Extraction and partition of ochratoxin from rice and coffee medium

For growth of toxicogenic strain in rice and coffee medium 35% moisture content were maintained. After the growth of mycelia for 15 days at room temperature, the mycelial broth was taken into a blender jar and extract with methanol:water(60:40) was added and the contents were blended for 3 min at high speed and filtered through Whatman No.1 filter paper. This filtrate was collected and 9 ml of saturated NaCl along with 15 ml of hexane was added and shaken for 5 minutes. Now this mixture was added to separating funnel and the lower aqueous phase was taken into another separating funnel. To this 10 ml of chloroform was added and then take the chloroform layer in another separating funnel to this 1.8 g cupric carbonate was added and again it was separated through separating funnel followed by this it was passed through bed of sodium sulphate column for removal of moisture content. This resulting fraction was evaporated for 2-3 ml in hot air oven at 40 °C for 5-6 hrs.

Quantification of ochratoxin using High-Performance Liquid Chromatography with fluorescence detector (HPLC-FLD)

Ochratoxin extraction with immuno-affinity clean-up

The dried chloroform extract were mixed with PBS buffer. Immunoaffinity columns (IAC, R-Biopharm Rhône Ltd., Glasgow, UK) were used for sample purification and ochratoxin extraction. Diluted filtrate (20 ml) was passed through IAC at approximately 0.6 ml/min by gravity. A slow flow rate was necessary for capturing ochratoxin by their antibodies in IAC, as recommended by the manufacturer. Then IAC was dried by passing air (syringe) for 10s. ochratoxin were eluted from IAC by 0.5 ml methanol then 0.5 ml water (Double distilled water) accompanied by back flushing. Air was passed through the column using a syringe to collect the last few drops. Collected elutes were clarified by a disposable filter membrane (sterile 0.45 µm) and stored in autosampler amber vials (Agilent Technology) at 4°C prior to injection into HPLC system.

Detection of ochratoxin by HPLC-FLD

A reversed phased (RP) HPLC procedure was used for ochratoxin determination in sample.The method described by (Zhu *et al.*, 2013) was followed with few modifications.A 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used for quantification of ochratoxin. The mobile phase consist of (A) 0.1% formic acid in water and (B) Acetonitrile, in the ratio of A(40):B(60)with a flow rate of 1 ml/min. Level of detections (LOD) of OTA was established by practical experimentation as being 0.5 µg/kg. Filtered samples (20 µl) were injected into system through the autosampler and ochratoxin signal were detected with FLD at excitation of 333 nm and 455 nm emission. The data of three replicates were acquired and analyzed with the Agilent Data Handling Chemstation3.

Spiking experiment

For accuracy of the experiment, spike analyses of the negative control fungal strain extract were done at 2 different levels of 2 and 5 ppb. The spiked samples were analyzed for recovery.

Results and Discussion

Ochratoxin producing fungal strain

The toxicogenic strain MTCC 4643 obtained from MTCC, Chandigarh, India. This strain was propogated on CYA media, which produced ochre colorcolonies on the plate. Microscopic characterization was done on 10x, 40x and 100x. It shows presence of large size conidia in the form of globose vesicles at the end of conidiophores.

Detection of ochratoxin production

Primary detection was done for ochratoxin production on synthetic media YES broth and it was incubated for 15 days at room temperature in static condition. As the incubation time increases after 7 days, the medium developed an ochre white colony; this

was due to production of secondary metabolites in the broth. As the fungal colony growth approaches to 15 day it, was extracted with chloroform and then subjected to thin layer chromatography for qualitative detection of it. The TLC plate shows presence of blue fluorescence in UV-transillumiator at long UV wave, which indicates presence of ochratoxin A in the sample. The rf value of standard were matches with that of MTCC 4643 Fig. 1.

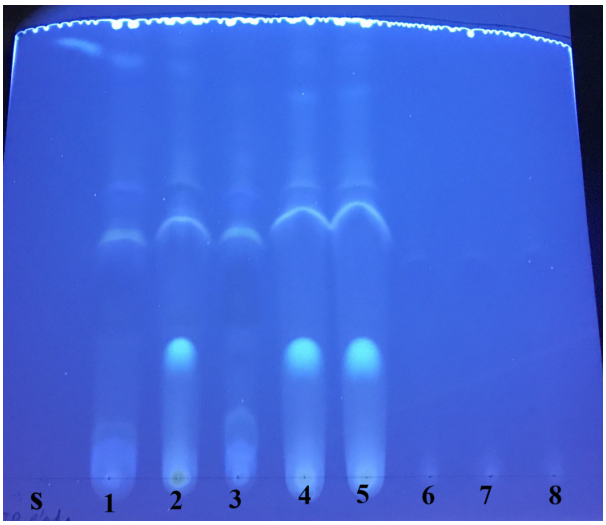

Fig. 1. Representative TLC image at long wave UV light cotaining of ochratoxinstandard 10 ppb in S lane of 50 µl volume; 4643 strain extract in chloroform 50 µl volume in lane 1; 4643 strain extract in chloroform 20 µl volume in lane 2; 4643 strain extract in chloroform 5 µl volume in lane 3; 4643 strain extract in chloroform 15 µl volume in lane 4; 4643 strain extract in chloroform 20 µl volume in lane 5; lane 6, lane 7 and lane 8 contain negative control

Ochratoxin recovery by HPLC-FLD

Fig. 2 shows HPLC chromatogram and elution times of Ochratoxin standard at 4.211 min. Recovery of ochratoxin form spiked samples (blank) wasas shown in Table 1. Total ochratoxin recovery was 95.68% and 96.11% at spiked concentrations of 2 and 5 µg/kg, respectively. Those percentages were

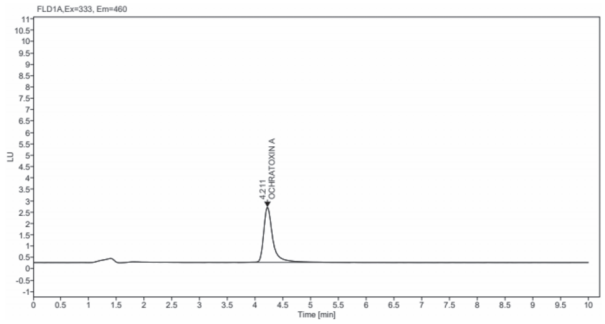

Fig. 2. Representative chromatogram of ochratoxin standard 10ppb

within acceptable values for ochratoxin of the AOAC which shows acceptable range between 70-120% (Trucksess *et al.*, 2011). The LOD for analysis was found to be 0.5 µg/kg and LOQ was 1 µg/kg.

Comparative quantification of ochratoxin in rice, coffee and SKMY medium

In the present study, visible growth of MTCC strain 4643 *A. ochraceous* in rice grain medium and coffee medium with 35% moisture content was denser at 15 days as compared at day 7. Mycelial growth at 15 day grown deeper ochrecolor as compared to day 7. *A. ochraceous* formed only Ochratoxin A at room temperature represented in Fig. 3. Ochratoxin B and Ochratoxin C were not detected at room temperature throughout the storage period. The ochratoxin production detected by HPLC-FLD was shown presence of high amount of ochratoxin in coffee medium as compared to rice medium and synthetic media YES. The amount of ochratoxin in rice medium was found to be 739.79 µg/kg represented in Fig. 4, in coffee medium it was found to be 823.74 µg/kg and in YES it was found to be 691.01 µg/kg.

Conclusion

This study suggests the effect of different media on production and quantification of ochratoxin. The level of toxin production varies between synthetic and natural media. The natural media for produc-

Table 1. Recovery(%) of aflatoxins(AFS) from samples spiked with two different concentrations

	Recovery (%) of Ohratoxin (OTA) from samples spiked with two different concentrations					
	Ochratoxin concentration (µg/kg)					
	level-1	obtained	% recovery	level-2	obtained	% recovery
OTA	2	2.01	100.5	5	5.04	100.8
	2	1.99	99.5	5	4.99	99.8

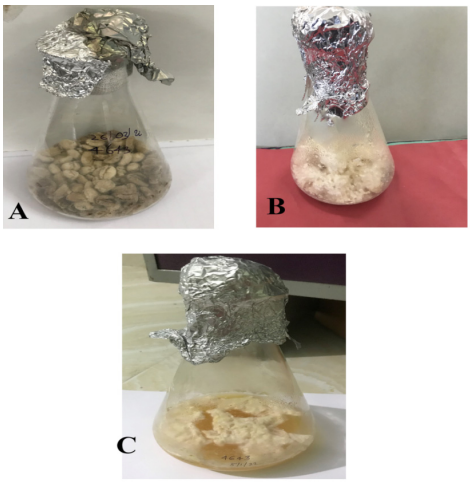

Fig. 3. Ochratoxin production in different medium: A - represents ochratoxin production in coffee medium; B - represents ochratoxin production in rice medium; C - represents ochratoxin production in YES medium

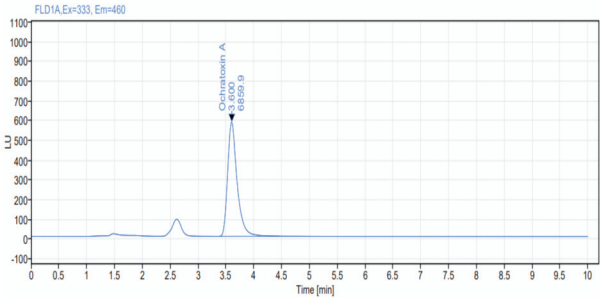

Fig. 4. Representative chromatogram of ochratoxin production of 4643 in coffee medium

tion of toxin are cost effective and easy to dispose as compared to synthetic media. In this study the coffee medium with 35% moisture content shows high production of ochratoxin as compared to other natural and synthetic medium.

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