

## EFFECT OF TEMPERATURE ON OCHRATOXIN A PRODUCTION IN COMMON CEREALS BY *ASPERGILLUS* SPECIES

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### ABSTRACT

Ochratoxin A (OTA) is a naturally occurring fungal metabolite produced by some species of *Aspergillus* and *Penicillium*. OTA being nephrotoxic, carcinogenic, hepatotoxic and immunosuppressive imparts many human and animal health hazards. In this study OTA production was observed at different temperatures (5°C, 10°C, 20°C, 30°C and 40°C) in three substrates (corn, rice and wheat) by *A. ochraceus* and *A. sulphureus*. These two fungal species were grown on aforementioned substrates for three weeks separately. It was quantified by High Performance Liquid Chromatography (HPLC) using the florescent detector. The samples were considered positive for OTA if the peak was obtained at the retention time of 6.34 minutes. OTA was produced at all studied temperatures and in all substrates but the maximum OTA production was observed ( $1.87 \pm 0.021$  µg/g) at 30°C in corn by the *Aspergillus ochraceus* where as it was minimum at 5°C in wheat by both species i.e. *A. ochraceus* ( $0.19 \pm 0.021$  µg/g) and *A. sulphureus* ( $0.09 \pm 0.046$  µg/g). Our results indicate that production of OTA by both species of fungus *A. ochraceus* and *A. sulphureus* depends upon temperature and substrate and the production in cereals was significantly higher than the maximum permitted level in cereals (5ng/g) as described by the European Union regulation commission and other agencies in the world.

**Key words:** Ochratoxin A; Temperatures; Cereals; *Aspergillus* species;

### INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species from *Aspergillus* and *Penicillium* genera. It has been extensively found in food items like grains, bread, nuts, spices, coffee, beer and wine, high levels in animal feedstuff (Otteneder and Majerus, 2001; Wangikar *et al.* 2005) and in animal meat (Singh *et al.* 1990). *Aspergillus ochraceus* is known to be the best for OTA production but *A. sulphureus*, *A. ostianus* and *A. sclerotiorum* are also considered the source of OTA production in cereals (Haggbloom and Ghosh, 1985). The most suspected foods susceptible to be contaminated by OTA are essentially domestic and imported cereals in North African countries (Hesseltine *et al.* 1979). OTA was first time isolated from the *Aspergillus ochraceus* as a secondary metabolite (Van der Merwe *et al.* 1965). OTA is one of the most important mycotoxin of human health concern. In our recent study on *in vitro* reported that oxidative DNA damage, measured by Comet assay, compares with the ability of OTA to induce micronuclei in both TK6 human lymphoblastoid cells and Chinese hamster ovary cells (CHO-K1-BH<sub>4</sub>) was observed (Ali *et al.* 2011). Consumption of OTA contaminated food from plant and animal origin could cause adverse effects on human health. It is implicated in a diverse range of pathological effects in poultry (Hassan *et al.* 2012). Based on

experimental animal study, the International Agency for Research on Cancer has classified OTA as a possible human carcinogen (IARC, 1993).

OTA is gaining a great attention due to its hazardous nature to animals and human health (Abarca *et al.*, 1994). Human is exposed to OTA directly by the consumption of contaminated cereal food products and their derivatives (plant sources) as well as derived from meat (animal sources) consumed the OTA contaminated feed (Czerwiecki *et al.* 2002; Duarte *et al.* 2010). OTA has been detected in blood and breast milk due to the ingestion of contaminated food (Grosso *et al.* 2003). OTA has been detected in bladder cancer patients at concentrations ranging from 0.03 to 3.41ng/ml and in healthy individuals at concentrations of 0.04-1.25 ng/ml from Pakistan (Aslam *et al.* 2008). It has wide range occurrence in food commodities, but the frequent contamination of OTA is determined in cereals throughout the world which is the main source of food and feed for human and animals that becomes cause of a continuous exposure of OTA to human (Radic *et al.*, 1997). OTA production has been reported higher in corn and wheat than in liquid media (Ciegler, 1972). The tolerance level of OTA intake has been established by the European Union and maximum permitted limit of OTA is 5ng/g in raw cereals and 3ng/g in processed cereals (Commission Regulation EC, 2005; Creppy *et al.*, 1995).

The growth of three strains of *Aspergillus ochraceus* and OTA production on barley grains was

observed with the effect of temperature and water activity. Maximum quantity of ochratoxins produced at the water activity treatment (0.98  $a_w$ ) after a three-week of incubation period and at temperature 25-30°C. The amount of Ochratoxins production is varied considerably with concentrations from 1.7 to 12,949 ppm, depending upon the availability of temperature and water activity ( $a_w$ ) (Scheuer and Leistner 1985). For analysis of OTA, extraction, clean up, confirmation and quantification are involved. OTA can be extracted from cereals by using a mixture of water and organic solvents (WHO – IPCS, 1990).

The aim of this work was to find out the production of OTA by two isolates of *Aspergillus* (*A. ochraceus* and *A. sulphureus*) at different temperatures normally persist in Pakistan throughout the year. The study was designed to observe the Ochratoxin A production by these fungal strains at different temperatures (5°C, 10°C, 20°C, 30°C and 40°C) in common cereals (corn, rice and wheat). Almost a very little data is available from Pakistan about the OTA production. This study will provide sufficient information on OTA exposure in Pakistan. However there is much need of awareness to avoid the people from this harmful contamination.

## MATERIALS AND METHODS

**Chemicals and materials:** All chemicals used in this study were HPLC grade of Merck brand (obtained from the local market Faisalabad, Pakistan). In all analytical steps double distilled deionized water was used. The ochratoxin A standard solution of concentration 10.07 µg/ml in acetonitrile was purchased from Biopure, Austria (Cat.No.002023) and Immunoaffinity column (IAC) containing specific monoclonal antibodies bound to a solid support material for OTA clean-up were purchased from the Romer lab, Rawalpindi, Pakistan office. Two ochratoxin A producing isolates *Aspergillus ochraceus* and *Aspergillus sulphureus* were obtained from National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan.

**Preparation of inoculums and media:** Three commonly used cereals; corn, rice and wheat were used in this study. 50g of fresh whole grain cereals (corn, rice and wheat) were taken in three sterilized 500 ml Erlenmeyer flasks. Equal quantity of fungal spores was inoculated in all flasks from one week old refreshed potato dextrose agar (PDA) slants. 3ml of 0.05% tween 80 was added to each slant/test tube and the spores were harvested by gentle scraping with sterile inoculation loop. The spores were suspended in solution form. Then 1ml of each fungal spore suspension was added to each flask containing substrates under sterile conditions and the contents of the flasks were thoroughly shaken to achieve the equal

distribution of the spores. The flasks were incubated in five separate incubators at desired temperatures of 5°C, 10°C, 20°C, 30°C and 40°C for three weeks. The flasks were shaken after each five hours to break the mycelial growth throughout the experiment (Trenk *et al.*, 1971).

**Extraction and IAC clean-up:** The substrates samples were analyzed for ochratoxin A production according to the method of Romer N° MY8402s (Romer Labs Inc., 1994) with some modifications. After three weeks of incubation, 25 gm of substrates (corn, rice and wheat) from each flask was ground to get the homogenous mixture. To each substrate sample, 100 ml total solution (84 ml acetonitrile and 16ml distilled water) was added and the resulting mixture was blended for three to five minutes at high speed with Osterizer blender. Then the suspensions were filtered twice with Whatman No. 4 filter paper to get clear filtrates. 7ml was taken from each filtrate, in a test tube and acidified with 70µl acetic acid. These acidified extracts were cleaned by passing through the immunoaffinity column (OchraStar™). 4ml of the resulting clean extracts were taken in glass ampoules and evaporated to dryness under the gentle nitrogen stream. The dried material was dissolved in acetonitrile and stored at 4°C until HPLC analysis, was performed. Five working solutions were prepared from the standard solution (10.07µg/ml) with the concentrations of 0.015, 0.025, 0.050, 0.10 and 0.20 µg/ml. Then the limit of detection (LOD) was observed that was 0.025µg/ml after the optimization (Bayman *et al.*, 2002)

**Analysis of ochratoxin A by HPLC-FLD:** The OTA production was detected and quantified by HPLC apparatus (Shimadzu, Japan) having loop injection of 20µl and equipped with fluorescence detector (Konik, model 403) (excitation wavelength 333nm and emission wavelength 460nm), using the C<sub>18</sub> chromatographic column (Sigma-Aldrich, USA). Three injections of 20µl volume of each sample were loaded manually. The mobile phase was (acetonitrile- water- acetic acid, 57: 41: 2, v/v) pumped through the heated column (30 °C) with flow rate of 1 ml/minute using Shimadzu 10 AVP high pressure isocratic solvent delivery system (Kyoto, Japan). The extracts were considered positive if the peak was obtained at the same retention time as that of OTA standards i.e. 6.34 minutes as shown in Figure 1. OTA was analyzed on the basis of fluorometric response of HPLC as compared with a calibration curve generated from an OTA standard. The limit of detection (LOD) was estimated 0.025 µg/g based on a signal-to-noise ratio of 3:1 and the limit of quantification (LOQ) was 0.1µg/g.

**Recovery Analysis:** 5 ml stock solution of OTA in acetonitrile was prepared having a concentration of 5 µg/ml for the study of recovery purpose. 25g of each OTA-free sample of corn, rice and wheat contained in a 250-ml Erlenmeyer flask was spiked with an equivalent

to 5, 10 and 20  $\mu\text{g}$  OTA  $\text{kg}^{-1}$ . Analysis of the blank sample was performed and spiking of OTA was carried out in duplicate. After leaving it for 15 hours to evaporate the solvent, extraction solvent was added and the OTA concentration was analyzed using the procedure as described above. Recovery %age was calculated for each sample (99.76% for corn, wheat 98.86% and 96.99%). Observed data was subjected to analysis of variance (two ways) using Statistix 8.1 software for the comparison of OTA production among the substrates and temperatures.

## RESULTS AND DISCUSSION

High Performance Liquid Chromatography Performance was used both for the qualitative and quantitative analysis of OTA. All peaks obtained on retention time 6.34 minutes were considered positive as optimized that of OTA standards (Figure 1). Figures-2 and 3 indicates the quantity of OTA production by both *Aspergillus ochraceus* and *Aspergillus sulphureus* respectively in three cereals on different five temperatures. Maximum OTA ( $1.87 \pm 0.021 \mu\text{g/g}$ ) production was observed in corn by *A. ochraceus* at 30°C temperature (Figure 2). At 40°C temperature, maximum OTA production ( $1.38 \pm 0.015 \mu\text{g/g}$ ) was noted in corn and other substrates. Then at 20°C temperature, less OTA ( $1.27 \pm 0.026 \mu\text{g/g}$ ) was produced as compared to high temperatures and this production was gradually decreased ( $0.76 \pm 0.040 \mu\text{g/g}$  and  $0.37 \pm 0.032 \mu\text{g/g}$ ) with the decrease in temperature 10°C and 5°C respectively (Figure 2). As concerned to substrates, corn produced maximum OTA and then rice and wheat.

Production of OTA was dropped below and above 30°C that shows the production is temperature dependent and 30°C was the most favorable temperature to produce significant quantity of OTA as compared to other temperatures. In this study effect of temperatures was observed for OTA production and moisture level was remained fixed for the maintenance of the natural state of cereals as being used for processing of food and feed as grain moistures affect more in OTA production than temperature (Monnet-Tschudi *et al.* 1997). *Aspergillus sulphureus* was used to assess the OTA production at all study temperatures and in all substrates but the concentrations were lower as compared to *A. ochraceus*, although the levels of production are in the same order as by *A. ochraceus* i.e. corn, rice and then wheat (Figure 3). It also represents the maximum OTA production at the same temperatures and in same substrates as described for *A. ochraceus* in Figure 2. Keeping the temperature, fungal strain and incubation period constant, the difference in OTA production was also noted using different substrates. The corn produced maximum level of OTA at all given temperatures as compared to other two substrates (Figure 2 and 3). The quantity of ochratoxin A produced by the same fungal strain in

different substrates is different. These findings are in accordance with the studies performed earlier (Payen *et al.* 1983; Alvarez *et al.* 2004).

Some fungal strains of a genus produce mycotoxin but other strains of same genus do not produce mycotoxin. The isolates used in this study produce measurable amounts of OTA at all temperatures and in all substrates. However another study reported that no OTA was produced by *A. ochraceus* (IMI 132429) at 10°C in autoclaved barley (Elmholt and Rasmussen, 2005). In Haggblom and Ghosh (1985) study, high concentration ( $4880 \pm 42 \mu\text{g/L}$ ) of OTA at 25°C in autoclaved barley using the *A. ochraceus* (589.68?) in the presence of glutamic acid was determined that match somewhat with these results in sense of production of OTA at 30°C by *A. ochraceus*. In this study OTA produced in all selected parameters (temperatures, cereals and fungus) was significantly higher as compared to the maximum tolerance limit for OTA in cereals (5ng/g) as fixed by the European Community Regulation no. 472/2002 (Commission Regulation EC, 2005; Creppy *et al.* 1995). The most ambient temperature for mycotoxin production is 25-30°C (Scheuer and Leistner, 1985) and in Pakistan this temperature ( $30 \pm 5^\circ\text{C}$ ) remains more than half of the year in most parts of the country. Most of the time storage conditions are not bothered for commodities/grains. These all conditions encourage OTA production through air born fungal infestations. The OTA was first time observed in corn seed in USA and also has been extensively observed in other cereals e.g. wheat, barley, rice and sorghum (Araguas *et al.* 2005). In another study, OTA was also observed with an average of 55, 96, 44 and 117  $\mu\text{g/kg}$  in wheat, barley, rice and sorghum respectively (Wood *et al.* 1997). A sufficient literature is available regarding OTA production by different fungal strains, temperatures and substrates, however there are fewer studies available from Pakistan on this issue. In African countries like Tunisia and Egypt, the people suffering from nephropathic problems can be considered with the consumption of contaminated domestic and imported cereals such as wheat, sorghum, olives, poultry products, and spices (Grosso *et al.* 2003). Probably two million people in Morocco are reported with chronic kidney diseases including chronic renal insufficiency and chronic interstitial nephropathy without any etiology. But later on survey illustrated that the Moroccan population was exposed to OTA and 60% of the Moroccan human plasma samples were positive for OTA with an average concentration of 0.29ng/mL (Filali *et al.* 2002).

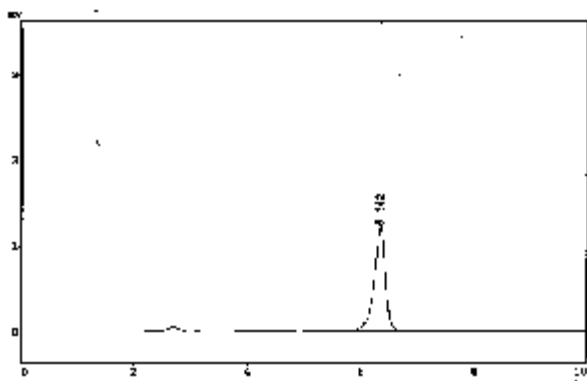
OTA toxicity is of great concern in Pakistani population. OTA in the blood of urinary bladder cancer patient has been detected in the range of 0.03 to 3.41ng/ml while in the healthy individuals the detected concentration was 0.04-1.25ng/ml (Aslam *et al.*, 2008). There is no mention of the source through which these

individuals were contaminated, so there is a need to make an elaborated study on every source through which the human can get this mycotoxin infection and our study is an effort to achieve this goal.

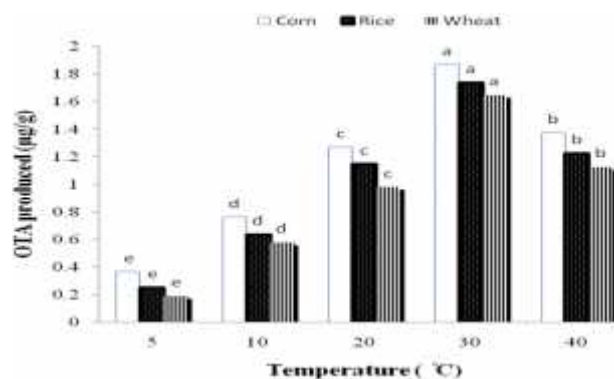
The present study was designed by keeping in mind the environmental conditions, fungal strains available and the storage conditions of country. Cereals, particularly the wheat, rice and corn are commonly used food crops all over the world. These cereals also share a major food sources in Pakistan especially the wheat and rice. Every year the production of wheat and rice is going increase as per consumption and Pakistan stands on 6<sup>th</sup> in the world (Economic Survey of Pakistan 2008-2009). Likewise in rice production, Pakistan is on 12<sup>th</sup> position with 4.5 million metric tons (www. NationMaster.com). The results indicate the production of ochratoxin A in commonly consumed cereals by the two fungal isolates. By adopting some suitable preventive measures, the production of OTA in stored grains can be avoided. As presence of OTA in food and feed is a health hazard, cereal storage should be done under situations which can restrict fungal growth and the consumption of contaminated foods should be avoided.

More rigorous studies are required on this critical issue of ochratoxin A to aware the people and concerned administration.

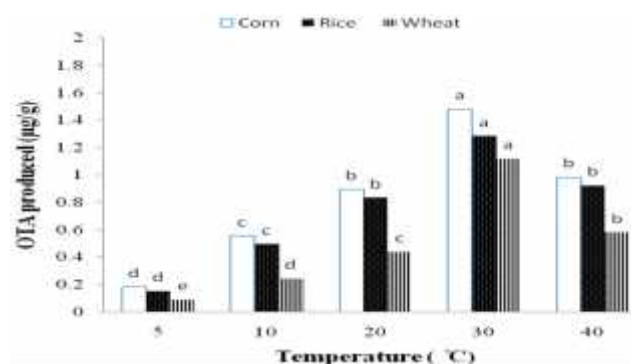
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**Figure-1: Chromatogram of OTA standard solution showing the Retention Time**



**Figure 2: Production of ochratoxin-A in different cereals and at different temperatures by *Aspergillus ochraceus***



**Figure 3: Production of ochratoxin-A in different cereals and at different temperatures by *Aspergillus sulphureus***

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