Isolation of *Aspergillus ochraceus* and Production of Ochratoxin in Coffee Samples

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**Abstract**

The occurrence of mycotoxins in foods and feeds has long been recognized as a potential hazard for human and animal health due to their severe toxic and carcinogenic properties. Among various mycotoxins, ochratoxin A (OTA) is the most hazardous one because of its serval contamination cases in the world. In this study contamination levels of *Aspergillus ochraceus* in coffee samples were assessed, where two strains of it were isolated from 65 coffee samples. They were confirmed by using Czapeck yeast extract agar (CYA). Fermentation was carried out for ochratoxin production at different moisture levels: 20%, 40% and 60% by using coffee as a substrate. The extract was assayed for the presence of ochratoxin by thin layer chromatography (TLC). Results showed that high production of this toxin (402 ppb) was obtained in coffee containing 60% of moisture after 19 days of incubation period with high load of fungal growth and the effect of roasting on coffee containing ochratoxin was observed where the level of it was decreased after roasting. It can be concluded that coffee substrate with high level of moisture favored good condition for the growth of *A. ochraceus* and ochratoxin formation.

**Key words**: coffee, *Aspergillus ochraceus*, OTA, CYA, TLC

**Introduction**

Ochratoxins are a group of mycotoxins produced by some *Aspergillus* species (mainly *A. ochraceus* but also by *A. niger*) and some *Penicillium* species, especially *P. verrucosum* (Pitt 1987). Ochratoxin A is the most prevalent and relevant fungal toxin of this group, while ochratoxin B and C are of lesser importance. The toxic effect of mycotoxins on animal and human health is referred to as mycotoxicosis, the severity of which depends on the toxicity of the mycotoxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects of other chemicals to which the individual is exposed (Peraica et al. 1999). According to Smith and Moss (1985) the most commonly acute affects of mycotoxin poisoning leads to deterioration of the liver and kidney functions, allergic response and immune suppression, which in extreme cases leads to death, whereas chronic effects include mutagenicity, teratogenicity and carcinogenicity (Mello & Macdonald 1997, Pitt 2000).

Ochratoxin A (OTA) structurally consists of a para-chlorophenolic group containing a dihydroisocoumarin moiety that is amide-linked to L-phenyl alanine (Khoury et al. 2010). It has been shown to exhibit nephrotoxic, immune suppressive, teratogenic and carcinogenic properties (Hohler 1998).

The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B), based on sufficient evidence for carcinogenicity in experimental animal studies and inadequate evidence in humans (IARC 1993). OTA frequently occurs as a contaminant in cereals worldwide and is also reported in coffee (Le-Bars & Le Bars 2000). *A. ochraceus* is thought to be the most important OTA producing mould in relation to coffee beans (Pitt 2000).

Coffee is relatively new crop in Nepal. It was introduced in Gulmi district of Lumbini zone at around 1937 AD. Nepal has potential for producing organic...
coffee due to the availability of soil with fragile nature and appropriate climate in the mid hill (Nepal 2006). In Nepal, studies on ochratoxin have not been carried out, where coffee beans are dried, the green beans contain 55% total polysaccharides, 8 to 15% fat depending on the variety, and around 11% protein, plus 10 to 13% water, alkaloids (including caffeine), and minerals (potassium, calcium, magnesium and phosphorus) which favors the growth of different types of fungus in presence of high amount of moisture. Fungi are significant environmental microorganisms especially in foods where they are responsible for spoilage, production of mycotoxins and, in some cases desirable bioconversions. Among them, ochratoxin A is nephrotoxic and carcinogenic to some animals and has been detected in different types of foods such as cocoa and cocoa products, coffee, dried fruits, cereals, wines, beer and others. A. ochraceus has been isolated from several green coffee samples originating from coffee producing countries. It is important to detect the contamination of coffee by ochratoxin producing fungi in Nepal.

Methodology

Isolation and identification of A. ochraceus

Fungi associated with green and roasted ground coffee beans samples were isolated according to the procedure given by International groundnut Aspergillus Flavus Nursery (IGAFN 1980).

For the isolation of mold associated with coffee samples, coffee beans were surface disinfected with 70% ethanol for 2 minutes and sodium hypochlorite for 3 minutes, washed with distilled water and dried in two layers of sterile filter papers. Subsequently, the beans were aseptically plated on potato dextrose agar (PDA) in order to isolate the associated internal micro flora. For powder samples, 10 g of coffee was taken and serial dilutions were performed and spread plate were done on PDA medium. The plates were incubated for 7 to 14 days at 28°C. After incubation, Aspergillus like colonies were sub-cultured on Czapek yeast extract agar (CYA) plates and thus obtained fungal colonies particularly A. ochraceus group were isolated and maintained on CYA. Identification was done on the basis of cultural and morphological characteristics described by Lactophenol Cotton Blue Staining Method (Aneja 2008, Pitt & Hocking 1997). The stock cultures of isolated colonies were maintained on CYA and PDA slants and preserved at 4°C.

Growth curve of Aspergillus ochraceus

Pure cultures of isolated strains of A. ochraceus were point inoculated on CYA plates separately and incubated at 28°C and growth of the cultures were measured. Taking measurement of diameter each day the growth curve of fungal isolates were drawn and their linear and maximum growth were determined (Aneja 2008).

Fungal culture and ochratoxin production

Fermentation was carried out in 500 ml Erlenmeyer flask containing 25g of Coffee. Initial moisture content of the substrate was determined by hot air oven method (KC & Rai 2007). Each substrate was inoculated with different strains of A. ochraceus by adjusting 20%, 40% and 60% moisture content with addition of water and incubated at 28°C for 20 days. During this time interval the level of ochratoxin and number of colony of mold was determined at different interval of time (Lindenfelser 1975, Bacon et al. 1973).

Extraction of ochratoxin

Extraction of ochratoxin from the solid substrate was carried out according to the Official Methods of Analysis (AOAC 1990) at every five days. The toxin was extracted by adding 12.5ml of water and 125ml of chloroform and allowed to shake for 30min on mechanical shaker. The mixture was filtered through Whatmann No.1 filter paper and the filtrate was again filtered through anhydrous sodium sulfate and chloroform was evaporated till dryness and toxins were identified on thin layer chromatography (TLC).

Quantification and Estimation of ochratoxin content

Quantification of ochratoxin was done by TLC using comparison of standards by visual technique. During loading about 500µl of acetic acid: benzene (1:99) was added to the extract residue and 5µl of sample extract was spotted on the TLC plate. Similarly, 5µl of standard ochratoxin having concentration 5ppm, 10ppm, 15ppm, 20ppm were spotted on the same TLC plate. The plate was then dipped into chromatographic tank containing solvent benzene: methanol: acetic acid (8:1:1) until the solvent moved up to the top of the plate (ie.up to 10-12cm) from the point of initial load. The TLC plate was dried and viewed in UV chamber at 365nm wavelength. Ochratoxin was observed as greenish blue color. The color intensity of the sample was matched with the color intensity of the standard solution. Then the concentration of ochratoxin was calculated (Helrich 1990).
Confirmation of the ochratoxin
The identity of the ochratoxin in the extract was confirmed by the presumptive test with alcoholic NaHCO₃. In this test alcoholic NaHCO₃ was sprayed on TLC plate, dried and observed under long wave length. Where fluorescence was changed from greenish blue to blue and increased in intensity (Helrich 1990).

Effect of heat on ochratoxin
Coffee samples that contained ochratoxin were taken and 25g of it was kept at hot air oven for roasting, the time was varied from 12 to 20 min, and the roasted colour varied from light medium to dark. The differences in OTA reduction between different levels of roasting was determined to see the effect of heat on the toxin (Vander Stegen et al. 2001).

Results and Discussion
Of 65 coffee samples, two samples showed the presence of A. ochraceus. These samples also contained different types of fungi. All together 53 samples of coffee beans were contaminated with different types of fungi such as A. flavus, A. niger, Mucor spp, Rhizopus spp. Penicillium spp, Fusarium spp.

A. ochraceus spp. isolated from coffee beans which were collected from Argakhachi and Kathmandu valley. Mostly fungi were isolated from coffee beans; among 12 samples of coffee powder only three samples were contaminated with fungi.

All the macroscopic characteristics of the isolates were similar to that of the A. ochraceus indicating that the isolates are most likely the strains of A. ochraceus.

确认的黄曲霉毒素
黄曲霉毒素在提取物中的身份通过使用含酒精的NaHCO₃的假定测试得到确认。在这种测试中，含酒精的NaHCO₃被喷在TLC板上，干燥后并在长波长下观察。荧光从浅绿蓝色变为蓝色并增加强度（Helrich 1990）。

热效应对黄曲霉毒素的影响
将含有黄曲霉毒素的咖啡样品取样，25g的样品在热空气烤箱中烘烤，烘烤时间为12至20分钟，烘烤后颜色从浅色到深色不等。不同水平烘烤差异被确定，以观察热量对毒素的影响（Vander Stegen et al. 2001）。

结果与讨论
从65个咖啡样本中，两个样本显示了A. ochraceus的存在。这些样本还含有不同类型的真菌。总共53个咖啡豆样品被不同类型的真菌污染，如A. flavus, A. niger, Mucor spp, Rhizopus spp, Penicillium spp, Fusarium spp。

A. ochraceus spp.从收集于Argakhachi和Kathmandu山谷的咖啡豆中分离。大多数真菌是从咖啡豆中分离的；在12个咖啡粉样品中，只有3个样品被真菌污染。

所有分离的菌株的宏观特性与A. ochraceus相似，这表明分离的菌株很可能是A. ochraceus的菌株。
Enumeration of *A. ochraceus* (AO₁ and AO₂) and detection of ochratoxin during fermentation

During the fermentation of the coffee sample at different moisture content, growth of fungi and the level of toxins were determined at the interval of 5 days. For the enumeration of fungi, one gram of sample was taken from each of the fermenting flask and dilution was performed up to 10⁻⁶. Ochratoxin was detected by taking 25 g of sample according to AOAC protocol.

The moisture content showed a marked influence on growth of *A. ochraceus* and ochratoxin A production. High numbers of *A. ochraceus* were present at 60% moisture after 5 days of incubation, in case of both strains, where the number of strain AO₁ was found to be log8.6 and AO₂ was found to be log8.76. No more variation was found in growth of *A. ochraceus* in 40% and 60% moisture content.

After 15 days of incubation the growth of *A. ochraceus* was at low level at 20% moisture in comparisons to 40% and 60%. The load of organisms was slightly in decline phase after 20 days of incubation. Similarly, detectable amount of ochratoxin A was produced after 4 days of incubation at 60% moisture, whereas ochratoxin A was detected after 9 days of incubation in 20% and 40% moisture content. Maximum level of toxin was produced at 60% moisture content after 19 days of incubation.

The high level of toxin produced by AO₁ strain was 400ppb in 60% moisture followed by 215ppb and 100ppb in 40% and 20% respectively, whereas maximum ochratoxin produced by AO₂ strain was 402ppb at 60% moisture followed by 210ppb and 100 ppb in 40% and 20% moisture respectively.

Effect of roasting on ochratoxin A level in coffee beans

Coffee sample which consisted ochratoxin was roasted for different interval of time and the content of toxin was determined according to AOAC. The effect of roasting was observed by determining the amount of ochratoxin. The result obtained is depicted below in Fig 6.
The growth curve (Fig. 1) of fungal isolates AO₁ and AO₂ shows linear growth pattern. The maximum size of fungal isolates AO₁ and AO₂ were obtained on 13th day which were 85mm and 86mm respectively. AO₂ had larger colony size than AO₁.

The optimum condition the growth of A. ochraceus and production of ochratoxin was 60% moisture content, where the isolated strain was inoculated in coffee sample at different moisture content. High yield of ochratoxin was obtained after 19 days of incubation in 60% moisture content. According to Northolt et al. (1979) A. ochraceus had the ability to grow at water activity (a_w) as low as 0.79 and reported optimum a_w for growth was 0.95. The minimum a_w for toxin production by A. ochraceus varied from 0.83 to 0.87 with an optimum of 0.99.

The level of ochratoxin was decreased by 50% after roasting the coffee beans till they turned dark color. According to Mounjouenpou et al. (2012) this reduction may be due to the formation of brown compounds such as melanoidins due to Maillard reactions. These, highly reactive compounds should be responsible for ochratoxin A complication observed. The intensity of the formation of these brown products is a function of the degree of roasting: This explains the low rate of complication observed with light roasted coffee. Suarez-Quiroz et al. (2005) suggested that OTA was masked by reactions with the substrate during roasting. The absence of OTA in green coffee beans is therefore the best guarantee of safety.

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Reference


