Proceeding of 2nd AFSSA Conference on Food Safety and Food Security
August 15 – 17, 2014
Asian Food Safety And Security Association
ISSN: 2306-2150

The optimized condition of ochratoxin a production for reference material production

Sudarut Kaokaeng [1], Warapa Mahakarnchanakul[1], Thanapoom Maneeboon[2], Chananya Chuaysrinule[2] and Kanithaporn Vangnai[1]

[1] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkhen Campus, Bangkok, Thailand 10900.
[2] Scientific Equipment and Research Division, KURDI, Kasetsart University, Bangkhen Campus, Bangkok, Thailand 10900.

Email address:
sudarut_6@hotmail.com (Sudarut Kaokaeng), fagiwpm @ ku.ac.th (Warapa Mahakarnchanakul), rditpm@ku.ac.th (Thanapoom Maneeboon) and chananya29@gmail.com (Chananya Chuaysrinule).

Abstract:

Ochratoxin A (OTA) is a significant mycotoxin that causes nephrotoxicity in animal and human. Since toxin is contaminated in foods at very small amounts as nanogram per gram (ng/g), thus the precision and accuracy of toxin analysis is required. This research aims to produce OTA in maize in order to develop as OTA reference material. The optimum conditions for fungal growth and toxin production was investigated. Comparing the toxin production by 4 strains of *Aspergillus spp*., isolated from brown rice and 2 reference strains were done under various substrates, temperatures, moisture contents and incubation times. Preliminary results showed strains I1012/1, 2277, K6723, P15723 including *A. ochraceus* TISTR 3557 and *A.alliaceus* TISTR 3218 produced toxin well on MEA agar at 28 °C for 7 days, measured by TLC method. Confirmation test on laboratory media showed only I1012/1 had ability to produce toxin well on CCA and PDA as 172 and 559 ng/g. However when cultured on maize and rice all five strains grew well, but did not produce toxin. Similar results was found when inoculated *A. ochraceus* TISTR 3557 on three different types of maize, Suwan 5, ATS 5 and popcorn grain, high mycelium production but none of toxin production was found. Changing strain to *A. alliaceus* TISTR 3218 was done and found very well toxin production on maize and rice at 20 %, 30 % and 40 % moisture, 28°C for 14 days as 330-500 ng/g, and produce better on MEA as 2000 ng/g, detected by HPLC method. While at this mentioned conditions *A. ochraceus* TISTR 3557 did not produce toxin. In conclusion the optimal condition for OTA production by *A. alliaceus* TISTR 3218 was on maize 30 % moisture (a_w 0.97) incubated at 28 °C for 7 days which could produce as high as 3200 ng/g.

Keywords: Mycotoxin, *Aspergillus alliaceus*, OTA reference material

1. Introduction

Ochratoxin A (OTA) is significant mycotoxins among five mycotoxins that are considered to be important in human health [11]. OTA is mainly produced by some species of *Aspergillus* and *Penicillium*, particularly *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and *Penicillium verrucosum*. Although these moulds can easily contaminate foodstuffs, but occurrence of OTA in foods may depend on climatic condition. The critical factors that affect fungal growth on food commodities are temperature, moisture content and time [3].

Toxicity of OTA is well documented in many animal species and in 1993 The International Agency for Research on Cancer (IARC) has classified OTA as Group 2B, a possible human carcinogen [5, 10]. In animal models, OTA was shown to produce a wide toxicological effect, including nephrotoxicity and nephrocarcinogenicity, neurotoxicity and immunotoxicity [12]. Since OTA is often not rapidly removed from the body and significant amounts may accumulate in the blood and other selected tissues. In March 2001, the commission of the European Communities has set OTA maximum levels of 5 ppb for raw cereal grains (including raw rice and buckwheat) and 3ppb for all

processed cereal products derived from cereals (including processed cereal products and cereal grains intended for direct human consumption).

Commonly used analytical methods for the determination of OTA are high-performance liquid chromatography (HPLC) with fluorescence detection or thin layer chromatography (TLC) with a cleanup step [13]. In the development of new analytical methods for OTA the analytical methods must be fully validated particular if they are to be used for control, monitoring and risk assessment studies [2]. Thus, the reference materials (RMs) or (CRMs), the materials containing a known or certified content of analyte(s) along with its uncertainty, are essential tools in achieving comparability and trueness of analytical data. Aside from that, the use of (CRMs) is a major requirement for an accreditation according to ISO/IEC 17025 [6]. This research aims to produce OTA in maize in order to develop as OTA reference material. The optimum conditions for fungal growth and toxin production was investigated.

2. Materials and Methods

2.1. Growth and toxin production on media

Fungal strains I1 / 1012, 2277, K6723, P 1572 isolated from brown rice and two reference strains from Institute of Scientific and Technological Research (TISTR), *A. ochraceus* TISTR 3557and *A. alliaceus* TISTR 3218, were used in this study. The isolates were cultured on CCA, CY20S, PDA and MEA then incubated at 28° C for 7 days. The analysis for the amount of toxin OTA was done by Thin Layer Chromatography (TLC) (Camag TLC scanner 3, Switzerland).

2.2. Ability of the toxin production on culture media and grain

Fungal strains I1 / 1012, 2277, K6723, P15723 and *A. ochraceus* TISTR 3557were cultured on CCA, CY20S, PDA and MEA. Replicate plates were incubated at 28° C for 7days then the center of agar colonies were drilled, put into a test tube. Pipetted 5 ml of saturated solution of ethanol - formic acid (25:1) then the mixture was shaken for 5 min, filtered through filter paper No 1. Then pipetted 2 ml of filtrate into a dark bottle, dried with nitrogen gas then dissolved with 200 µl of benzene : acetic acid (99:1). The amount of OTA in the extract was analyzed by TLC-densitometry method

2.3. Culture preparation and inoculation

Spore suspension was prepared by culture fungal isolates on malt extract agar (MEA) at 28°C for 7 days, then added 10 ml and wiped the upper layer of media with sterile hockey stick. Pipette spore suspension and mixed with vertex. Maize 100 g were added with sterile water to achieve 20%, 30% and 40% moisture, placed in sterile plastic and inoculated with 10 ml of spore suspension. Growth was determined after maize was incubated at 28 °C and 35 °C and measured the diameter of colony at 7 days. The experiment was carried out twice with two replicates per conditions.

To compare OTA production on maize and rice, sterile water was added to maize and rice as 10, 20 and 30 ml of water to achieve 20%, 30% and 40 % moisture. Five isolates I1012/1, 2277, K6723, P15723 including *A. ochraceus* TISTR 3557, was chosen and mixed with 100 grams of maize and rice left overnight in sterile plastic, then autoclaved for 30 min before inoculated at 28°C and 35°C for 7 and 14 days.

2.4. TLC-densitometry detection of OTA

High performance TLC sheet was baked at 105 °C for 1 h then washed (predevelop) by using chloroform - methanol (1:1) which will be used mobile phase. Let solvent moves to the left of a sheet as 80 mm. When the plate is dry, immediately used to analyze, but if it is not used immediately plate should store in a desiccator at 30 ° C. Extract sample 20l was spot onto TLC plate added develop solution of toluene - ethyl acetate - formic acid (6:3:1) used as mobile phase let solvent moves to 70 mm. Read plate with Dennis, wavelength of 333 nm using a mercury lamp as a light source. Then confirmed by spectroscopy photometer (Camag TLC scanner 3) at a wavelength of 200-500 nm using fluorescent as a light source. Standard solution was prepared at concentrations of 2.5, 7.5, 10.25, 17.5 and 22.5 ng/g, respectively, and spot same time of sample detection.

2.5. HPLC detection of ochratoxin A

After 7 and 14 days of incubation, two replicates per conditions were blended and dried at 70 °C for 12 h, stored at 4°C until OTA analysis was carried out. 50g of sample was weighed and ground with 5 g of NaCl in blender jar, added 100 mL methanol: water (80:20), and blended at high speed for 1 minute. Extract was filtered through filter paper no. 4 and filtrate was collected in a clean vessel. Pipetted filtrate 10 mL and diluted with 40 mL PBS. Mixed well and filtered again through 1.5 µm glass microfiber and collected. 10 mL Filtrate was filtered through column (VICAM OchraTest^{TM}, USA) at a rate of 1-2 drops/second then the column was washed with 10 mL PBS containing 0.01% tween 20, and then rinse twice with 5 mL distilled water. OTA was eluted from the column using methanol (HPLC grade) as a solvent, at a flow rate of 1–2 drops per second. Each treatment was performed by HPLC (Water, USA).

3. Results and Discussion

Among five isolates from rice I1012/1 has shown the high ability to produce OTA when cultured on CCA and PDA at 28°C for 7 days (Table 1) comparing to other isolates. Reference culture *A. alliaceus* TISTR 3218 produced OTA better on CCA, CY20S, PDA and produced the highest amount of OTA as 2090 ng/g on MEA comparing to *A. ochraceus*, which was commonly isolated from maize. Then *A. alliaceus* was inoculated on maize and rice at different moisture contents and incubated for 7-14 days.

Table 1 *Concentration of OTA in agar (ng/g) extracted from each isolates cultured on laboratory media at 28 °C for 7 days*

Culture	OTA (ng/g)[c]			
	CCA	CY20S	PDA	MEA
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
A.ochraceus [a]	131.41 ± 0.63	-	300.28 ± 0.27	400.35 ± 2.76
A.alliaceus [b]	1678.00 ± 1.03	1350.85 ± 2.30	1245.85 ± 1.54	2090.00 ± 1.16
2277	-	-	94.36 ± 1.34	-
I1012/1	172.00 ± 0.23	-	559.00 ± 0.99	-
K6723	-	-	-	-
P15723	44.78 ± 0.15	-	0.68 ± 0.54	-

[a] *Reference culture A. ochraceus TISTR 3557,* [b] *Reference culture A. alliaceus TISTR 3218*
[c] *detected by TLC*

Fig. 1 showed *A. alliaceus* has potentially produced high amount of toxin production on both maize and rice at 20%, 30% and 40 % moisture, 28°C for 7 days as 260-400 ng/g, and 14 days as 300-400 ng/g, the selected condition *A. alliaceus* for OTA production was on 30% moisture maize and incubate for 7 days.

The growth and toxin production of *A. ochraceus* TISTR 3557 and four isolated from rice were observed on maize and rice at 20%, 30% and 40% moisture. Although all five strains shown the potential to produce OTA on laboratory media and grew very well on maize and rice but none of those produce toxin at any conditions (Table 2), including reference strain *A. ochraceus* TISTR 3557.

Similar results was found when cultured *A. ochraceus* TISTR 3557 on three different variety of maize, Suwan 5, ATS 5 and popcorn grain, high mycelium production was shown but none of toxin production was found (Table 3). Although maize was crushed, or added yeast extract as a nutrient to promote OTA production and this strain showed high growth on Suwan 5 and ATS 5 but none of OTA was found. Further study we decided to use reference strain *A. alliaceus* as the OTA producing strain to produce OTA reference material, and Suwan 5 was use as the material.

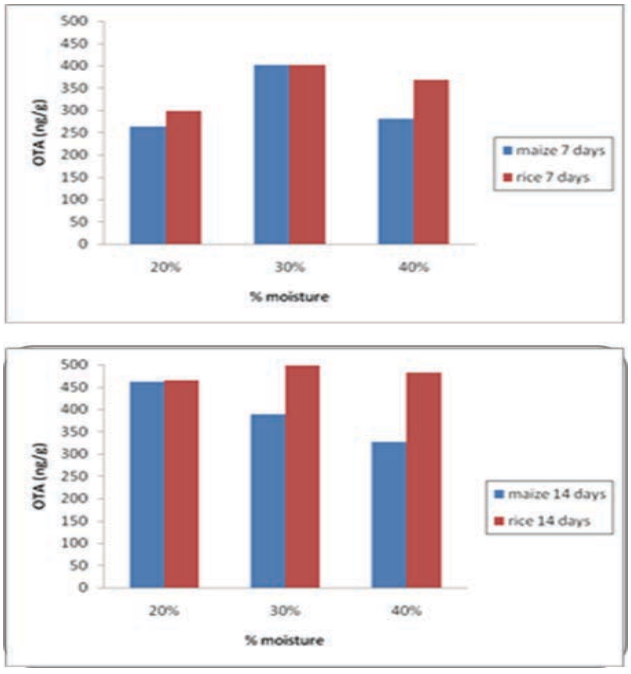

Fig. 1 *OTA concentration (ng/g) produced by A. alliaceus TISTR 3218 strains on maize and rice at different % moisture and incubation at 28 °C for 7 and 14 days*

Table 2 *Ochratoxin production by Aspergillus stains under various growth conditions*

Culture	Sample	% moisture (wet wt.)												OTA (ng/g)											
		20%				30%				40%				20%				30%				40%			
		28°C		35°C		28°C		35°C		28°C		35°C		28°C		35°C		28°C		35°C		28°C		35°C	
		7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14
A. ochraceus	Maize	+++[a]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-*	-	-	-	-	-	-	-	-	-	-	-
	rice	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-
I1012/1	Maize	++[b]	+++	++	+++	+++	+++	+++	+++	++	+++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
	rice	++	+++	++	+++	+++	+++	+++	+++	++	+++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
2277	Maize	++	+++	++	+++	++	+++	++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-
	rice	++	+++	++	+++	+[c]	+++	++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-

Culture	Sample	% moisture (wet wt.)												OTA (ng/g)									
		20%				30%				40%				20%				30%				40%	
		28°C		35°C		28°C		35°C		28°C		35°C		28°C		35°C		28°C		35°C		28°C	
		7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14
K6723	Maize	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-
	rice	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-
P15723	Maize	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-
	rice	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-

+++[a] = excellent mycelium production (71-100%), ++ [b] = good mycelium production (31-70%),
+ [c] = low mycelium production (0-30%),
** = less than limit of detection (LOD) 0.87 ng/g by HPLC/FLD detection*

Table 3. *OTA concentration (ng/g) produced by A. ochraceus TISTR 3557 on three different variety of maize at 30 %moisture incubation at 28 °C for 14 days*

Type of maize	Suwan 5		ATS 5		popcorn grain	
	growth	OTA (ng/g)	growth	OTA (ng/g)	growth	OTA (ng/g)
Grain	+++	-*	+++	-	++	-
Crushed	+++[a]	-	+++	-	++	-
Crushed: grain (1:10)	+++	-	+++	-	++	-
Yeast extract: grain(1:100)	++[b]	-	++	-	+[c]	-

+++ [a] = excellent mycelium production (71-100%), ++ [b] = good mycelium production (31-70%),
+ [c] = low mycelium production (0-30%),
** = less than limit of detection (LOD) 0.87 ng/g by HPLC/FLD detection*

Table 4*. OTA concentration (ng/g) produced by A. alliaceus TISTR 3218 on maize 30 %moisture at 28 °C for 7 days*

Toxin	Culture	20%	30%	40%
		OTA (ng/g)	OTA (ng/g)	OTA (ng/g)
OTA	*A. alliaceus*	1099.05 ± 131.03	3200.24 ± 61.43	908.14 ± 120.82

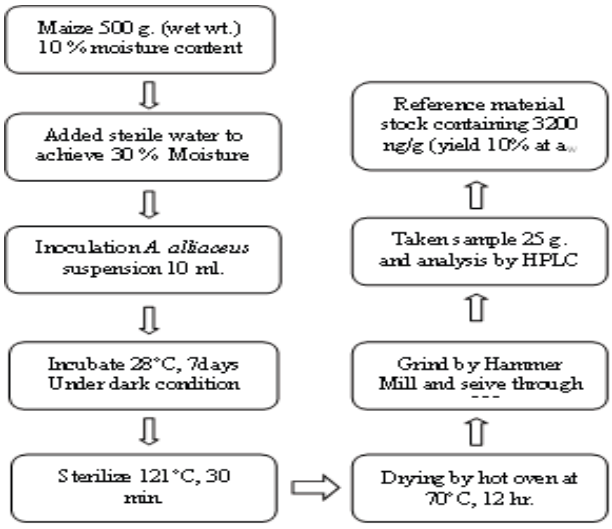

Fig 2*. Flow chart showing the step of OTA reference materials production*

Fig.2 concluded the scheme of OTA production in our laboratory which achieve the final product of OTA reference material containing 3200 ng/g (a_w 0.97) and has yield 10% from 500g maize.

This study detailed the optimum conditions for fungal growth and toxin production of A. alliaceus TISTR 3218. This study has shown the interactions of substrate, temperature and time to optimize the condition producing OTA and further scale up to produce the OTA reference material.

Our five isolates from brown rice and reference culture *A. ochraceus* TISTR 3557 could grow and produced OTA well when cultured on laboratory media MEA, while lost ability to produce toxin when cultured on maize and rice. Actually OTA producing fungal *A. ochraceus* is mostly used to study the production of toxins OTA, similar result was found that *A. ochraceus*, isolates from natural source such nut and fig, produced OTA less than (LOD) 0.01 µg/ml [4], while *A. alliaceus* isolated from the source produced substantial OTA as high as 30000 ng/g. Our reference strain *A. alliaceus* TISTR 3218 exhibit high OTA production up to 3200 ng/g on maize and thus at appropriate conditions for growth and OTA production, 30% and incubated at 28 °C, could be used in the manufacture of reference materials.

Optimal conditions for growth and OTA production according to Ali *et al.* (2013) showed that *A. ochraceus* , obtained from NIBGE Pakistan, could produce toxin well on corn, rice and wheat as 1600-1870 ng/g at 30 °C incubated for 3 weeks, while this strain produce less OTA production at 20 °C and 40 °C as 1000-1380 ng/g. OTA production decreases as temperatures increase [1, 8]. The different ability to produce toxin may differ from strain to strain. Individual fungal species also differ in their growth responses to the water activity (a_w) and temperature of the food such as grain [7].

4. Conclusion

Based on Bayman *et al*. (2001) suggested that future studies of ochratoxin production and contamination by the aspergilli should focus on *A. alliaceus* rather than *A. ochraceus* [4]. Studies of the *A. ochraceus* group are complicated by difficulties in distinguishing *A. ochraceus* from related species.

Corn grain (Zea maize) is an important food for large numbers of people in the developing world. Corn and products from corn are frequently contaminated with ochratoxin A (OTA) and associated with OTA producer fungi (Caldas *et al*., 2002). Apart from OTA producing fungal, corn have been concerned with the other contaminated fungal. *Fusarium* spp. are generally considered to be field fungi although this fungal require high water for growth, but they can sometimes grow in stored grain [9] while *Aspergillus* and *Penicillium* spp. are typical storage species that are able to thrive at relatively low water activities [7].

References

[1] Ali, R., Ismail, M., Mobeen, A., & Khan, Q.M. 2013. Effect of temperature on ochratoxin a production in common cereals by *Aspergillus* species. Journal of Animal & Plant Science, 23(5), 1316-1320.

[2] Araguas, C., Gonzalez-Penas, E., & Lopez de Cerain, A. 2005. Study on ochratoxin A in ceral-derived products from Spain. Journal of Food Chemistry, 92, 459-464.

[3] Arroyo, M., Aldred, D., & Magan, N. 2005. Environmental factors and weak organic acid interactions have differential effects on control of growth and ochratoxin A production by *Penicillium verrucosum* isolates in bread. International Journal of Food Microbiology, 98, 223–231.

[4] Bayman, Paul., James L, Baker., Mark A, Doster., Themis J, Michailides., & Noreen E, Mahoney. 2002. Ochratoxin Production by the *Aspergillus ochraceus* Group and *Aspergillus alliaceus.* Journal of American Society for Microbiology, 68, 2326-2329.

[5] IARC (International Agency for Research on Cancer). 1993. In "IARC Monographs on the Evaluation of Carcinogenic Risks to Homans: Some Naturally Occurring Substances; Food Items and Constiuents, Heterocyclic Aromatic Amines and Mycotoxins" 56. 489 IARC (Ed). Geneve.

[6] International Organisation for Standardisation. 1999. General Requirements for the Competence of Testing and Calibration Laboratories. ISO/IEC 1702517025. International Organisation for Standardisation, Geneva.

[7] Lee, H.,B., & Managan, N. 2000. Impact of environment and interspecific interactions between spoilage fungi and Aspergillus ochraceus on growth and ochratoxin production in maize grain. International Journal of Food Microbiology, 61, 11-16.

[8] Maren A, Klich., Sean, Tang., & David W, Denning. 2009. Aflatoxin and Ochratoxin Production by Aspergillus Species Under Ex Vivo Conditions. Journal of Mycopathologia, 168, 185-191.

[9] Pardo, E., Marı´n, S., Sanchis, V., & Ramos, A.J. 2004. Prediction of fungal growth and ochratoxin A production by Aspergillus ochraceus on irradiated barley grain as influenced by temperature and water activity. International Journal of Food Microbiology, 95, 79-88.

[10] Pena, A., Seirtova, M., Lino, C., Silveira, I., & Solich, P. 2006. Food Chem. Toxicol, (Chapter44).

[11] Polisenska, I., Pfohl-Leszkowicz, A., Hadjeba, K., Dohnal, V., Jirsa, O., Denesova, O., Jezkova, A., & Macharackova, P. 2010. Occurrence of ochratoxin A and citrinin in Czech cereals and comparison of two HPLC methods for ochratoxin A detection. Journal of Food Additives and Contaminants, 27, 1545-1557.

[12] WHO. 2001. Ochratoxin A. Safety assessment of certain mycotoxins in food. WHO Food Additives Series No. 47. Geneva: WHO, (pp 281-415).

[13] Zheng, Zhongming., Hanneken, Jonne., Houchins, Donna., King, Robin S., Lee, King., & Richard, John L. 2005. Validation of an ELISA test kit for the detection of ochratoxin A in several food commodities by comparison with HPLC. Journal of Mycopathologia, 159, 265-272.