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OCHRATOXIN A *„IN VITRO"* BIOSYNTHESIS BY THE *ASPERGILLUS OCHRACEUS* E'G ISOLATE

ABSTRACT: This paper deals with the biosynthetic capacity for ochratoxin A (OTA) production by *Aspergillus ochraceus* E'G isolate derived from *A. ochraceus* CBS 108.08 strain, during 2007. Preliminary analysis of fungal potential for the production of OTA were performed according to the modified method of Filtenborg et al. (1983). Toxin production was tested in the following liquid media: (i) glucose-peptone-yeast extract broth (GPY — pH 5.6), (ii) potato-dextrose broth (PDB — pH 6.9), (iii) yeast extract-sucrose broth (YES — pH 6.5), and (iv) YES broth supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O (YES^{Zn} — pH 6.5) after stationary and submerged cultivation. Dynamics of OTA biosynthesis was tested after the cultivation of *A. ochraceus* E'G on natural solid substrates, such as wet sterilized rice, corn and wheat grain. Cultivations were performed during different time periods (ranging from four days to few weeks) at different temperatures (ranging from 21°C to 30°C).

The presence of OTA was determined as follows: (i) in liquid media according to the method of Balzer et al. (1978) modified by Bočarov-Stančić et al. (2003), and (ii) in the solid substrates according to the Serbian official methods for sampling and analyzing of fodder (Official Gazette of SFRY, No. 15/87).

After the cultivation of *A. ochraceus* E'G isolate in liquid media, the highest yield of OTA (6.4 mg/l) was obtained after submerged cultivation in PDB (4 days, 128 rpm, 21—23°C). In the case of cultivation on solid substrates, the highest amount of OTA (800.0 mg/kg of dry matter) was recorded after several week long cultivation on wheat grain at 30 ± 1°C.

KEY WORDS: *Aspergillus ochraceus*, ochratoxin A, *in vitro* biosynthesis

INTRODUCTION

Ochratoxin A (OTA) is a potent kidney toxin which causes birth defects in test animals (rats, hamsters and mice). In terms of livestock and human health this mycotoxin is particularly important for the poultry and swine, because these monogastric animals lack the ability to degrade OTA rapidly, as compared to ruminants.

Ochratoxin A is the contaminant of a variety of plants and animal products. Although this mycotoxin is a world wide problem, its impact is the greatest in temperate regions where great amount of world's grain is produced and stored. Because of that, OTA has become a major concern to livestock producers, especially in Europe and North America. Once induced into feed of monogastric animals, it contaminates eggs, organs, fat, muscle tissue and blood (Abramson, 2008). The main sources of ochratoxin A in human diet are undoubtedly cereal products and swine products, although other commodities, such as coffee, wine, beer, cheese, poultry products, pepper etc. may also contain traces of this toxin (Gatti et al., 2003; Tjamos et al., 2004; Mahdavi et al., 2007). Medina et al. (2004) even hypothesized that bee pollen may constitute an important risk factor concerning the presence of OTA in the diet of consumers of that nutritious food.

A number of authors reported about the importance of OTA as one of the basic contaminants of fodder and feed components in the Republic of Serbia. During 1993—2003 period, Jakić-Dimić et al. (2003) analyzed 220 samples of fodder types for different growth categories of cattle and found that OTA and zearalenone were the most frequent contaminants. In 2004, in the samples of stored barley, taken from Kragujevac locality and intended for livestock nutrition, Škrinjar et al. (2007) recorded the presence of ochratoxin A in all samples, in the concentrations ranging from 0.54 to 7.3 μg/kg. According to Jurić et al. (2005), OTA occurrence is a serious problem in Serbia. In a 3-year period these authors analyzed 229 samples of feed components and found this mycotoxin in 50% of maize samples, and even 100% of sunflower pellets; the highest concentrations of OTA were 0.5—1.0 mg/kg.

Ochratoxin A can be produced by the number of fungal species belonging to the genera *Aspergillus* and *Penicillium*, but principal producers of this mycotoxin are *A. ochraceus* in warm regions of the world, and *P. aurantiogriseum* in temperate climates (Frisvald and Samson, 1991). As a rule, *Aspergillus* species grow in conditions of low water activity (min. 16%) and high temperatures, unlike typical field mycobiota, such as *Fusarium* species. Under laboratory conditions, they can grow in the range of 4.5—8.0 pH value, although the optimal pH for their growth is 5.5—7.5. Since the *Aspergillus* species, as well as other fungal species, are strictly aerobic, they generally require minimum 1—2% of oxygen for growth (Sinovec et al., 2006).

Accordingly, the aim of this paper was to examine the kinetics of OTA biosynthesis under laboratory conditions, and to complete the optimization of conditions for the OTA production by the newly isolated culture of *A. ochraceus* designated E'G.

MATERIAL AND METHODS

Microorganisms. *Aspergillus ochraceus* Wilhelm isolate E'G, derived in 2007 from CBS 108.08 strain, and CBS 108.08, known as ochratoxin A (OTA) producer, were investigated. The E'G isolate was obtained from the CBS 108.08 colony that showed segregation during 10-day growth on yeast extract (2%) sucrose (15%) agar (2%) with the addition of 0.23 mg/l $ZnSO_4$ x 5 H_2O (YESA^{Zn}, pH 6.5) (M ü c h l e n c o e r t, 2004) at 27 ± 1°C. The obtained isolate E' morphologically differed from the parent one: smaller conidial heads were observed (average diameter 173 μm compared with 244 μm of the original strain CBS 108.08). Also, the colour of the colony grown on potato-dextrose agar (PDA) was not typically ocher but rather green. After sowing of E' isolate on PDA plates and 10-day cultivation at 27 ± 1°C, the observed characteristics remained unchanged. After one year storage on PDA slants at 4—6°C, the E' isolate did not show any growth during the subcultivation on PDA plates at 27 ± 1°C, so the nutrient broth was used for enrichment and revivification of the E' culture. This procedure resulted in consistently olive green colony colour and smaller conidial heads during the cultivation on PDA at 27 ± 1°C, as well as at 30 ± 1°C. The obtained isolate was designated as E'G.

Preliminary analyses of fungal potential to produce OTA were performed according to the rapid screening method of F i l t e n b o r g et al. (1983) modified by B o č a r o v - S t a n č i ć et al. (in press) on the following media: YESA (2% yeast extract, 15% sucrose and 2% agar, pH 6.5), YESA^{Zn} (2% yeast extract, 15% sucrose, 0.23 mg/l $ZnSO_4$ x 5 H_2O, and 2% agar, pH 6.5), PPSA (2% peptone-1, 15% sucrose and 2% agar, pH 6.5), PPSA^{Zn} (2% peptone-1, 15% sucrose, 0.23 mg/l $ZnSO_4$ x 5 H_2O and 2% agar, pH 6.5) and PDA (potato-dextrose agar, pH 6.9).

Inoculations of liquid and natural solid fermentation media for testing kinetics of OTA biosynthesis were performed by 5 peaces (5 x 5 mm) of fungal material originating from Petri dish sowed with tested culture, and subcultivated for 7 days on potato-dextrose agar (PDA) at 27 ± 1°C.

Cultivation conditions. Toxin production was tested in the following types of liquid media: (i) broth with 5% glucose, 0.1% peptone-1 and 0.1% yeast extract, pH 5.6 (GPY), (ii) potato dextrose broth (PDB: 200 g/l of boiled sliced potato + 20 g/l dextrose, pH 6.9), (iii) broth with 2% yeast extract and 15% sucrose, pH 6.5 (YES), and (iv) broth with 2% yeast extract and 15% sucrose supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O, pH 6.5 (YES^{Zn}). Inoculated Erlenmayer flasks (500 ml) containing 100 ml of each medium were cultivated for 4 days on rotary shaker (128 rpm) at room temperature (21—23°C), and 10 days stationary at 27 ± 1°C in chamber. pH value was measured after the cultivation of the isolate.

Inoculated Roux bottles containing 50 g of sterilized cereal kernels, such as rice, corn, and wheat, wetted with 50 ml of sterile water, were cultivated at 30 ± 1°C in chamber for four weeks, or at room temperature (21—23°C) for 3 weeks. In the case of corn and wheat substrates, the samples for analysis were taken weekly during the cultivation period. The moisture content of the tested

natural substrates was determined after drying at 105°C until constant weight was not achieved.

All cultivations were performed in two replications.

Analyses of OTA. After the cultivation on rotary shaker, liquid cultures were filtered. Crude OTA extracts were obtained by the use of acetonitrile/water (90 + 10 v/v), according to Balzer et al. (1978) method modified by Bočarov-Stančić et al. (2003). The modification consisted of adding the culture filtrate up to 20% of anh. Na_2SO_4, as well as 20% of silica gel for column chromatography (Kieselgel 60 extra pure — MERCK) before toxin extraction. The rest of the analysis was done according to the original Balzer et al. (1978) method.

The samples obtained after the cultivation on cereal grains were dried during 24 h or more at 60°C until constant weight was not obtained. After the pulverization of dried samples, OTA was analyzed according to the Serbian official methods for sampling and analyzing of fodder (Official Gazette of SFRY, No. 15/87). After the cultivation on natural substrates, the yield of OTA was expressed as number of mg per kg of dry matter of the sample.

All analyses were done in three replications.

RESULTS AND DISCUSSION

Results of the present investigation are shown in Tables 1—3.

Tab. 1 — Screening of ochratoxin A production by *Aspergillus ochraceus* CBS 108.08 and E'G isolates

Sample designation	*Temper. (°C)*	*Days*	*Medium*				
			PPSA^{Zn}	PPSA	PDA	YESA	YESA^{Zn}
CBS 108.08	27 ± 1	10	+	—	+++	++++	++++
E'G	21—24	7	n.a.	n.a.	+	++++	++++
		10	n.a.	n.a.	+	++++	++++
		14	n.a.	n.a.	+	++++	++++
	27 ± 1	10	+	—	++	n.a.	n.a.

Legend: n.a. — not analyzed; — no biosynthesis; + low intensity of biosynthesis, ++ moderate intensity, +++ high intensity, ++++ very high intensity

The ability of *A. ochraceus* isolate E'G to produce ochratoxin A was tested simultaneously after 10-day cultivation at 27 ± 1°C in chamber, and after 14-day cultivation at room temperature (21—24°C). Isolate E'G showed almost the same toxicological profile as the parent strain CBS.108.08, with the exception of cultivation on PDA plates where it produced lower quantities of OTA than CBS.108.08 did (Table 1).

During the cultivation of E'G isolate at room temperature (21—24°C) the best results were achieved on YESA and YESA^{Zn} plates. Under the same temperature conditions it was observed that biosynthesis of the tested mycotoxin did not change over time.

Tab. 2 — Biosynthesis of ochratoxin A by *A. ochraceus* E'G in liquid media

Temper. (°C)	*Cultiv. conditions*	*Days*	*pH*	*Medium*	*Yield (mg/l)*
21—23	128 rpm	4	5.12	GPY	n.d.
27 ± 1	stat.	10	4.75		n.d.
21—23	128 rpm	4	6.50	PDB	6.40
27 ± 1	stat.	10	5.80		0.80
21—23	128 rpm	4	5.15	YES	0.08
27 ± 1	stat.	10	5.04		0.04
21—23	128 rpm	4	5.28	YES^{Zn}	0.06
27 ± 1	stat.	10	5.01		n.d.

Legend: n.d. — not detected (< 0.004 mg/l)

The production of ochratoxin A in liquid media was tested simultaneously after stationary and submerged cultivation in: (i) GPY — pH 5.6, (ii) PDB — pH 6.9, (iii) YES — pH 6.5, and (iv) YES^{Zn} — pH 6.5. The initial pH of the tested media was adjusted to these values, having in mind the ascertainment of Mühlencoert (2004) that ochratoxin A biosynthesis can occur between 5.5 and 8.0 pH. At the end of the cultivation period the decrease of initial pH was recorded in all cases, although the most outstanding change occurred after 10-day stationary cultivation in chamber (27 ± 1°C) (Table 2).

During the testing of OTA production by *A. ochraceus* isolates, Mühlencoert (2004) used different liquid media, including YES broth (initial pH 6.5) in shaken incubation flasks (120 rpm) at 25°C in the dark. This author observed that after onset of OTA biosynthesis (after 72 h) there was a steady increase of toxin concentration, which was accompanied, like in our experiments, by a drop of pH.

During the presented experiments in different liquid media, OTA production was not at all recorded in GPY broth, while in YES broth and YES supplemented with Zn only small amounts of the toxin were found mostly after submerged cultivation (0.08 mg/l and 0.06 mg/l, respectively) (Table 2). The highest amounts of ochratoxin A were observed after the cultivation of E'G isolate in potato-dextrose broth. Although the temperature applied during the submerged cultivation in PDB was lower (21—23°C) than during the stationary one (27 ± 1°C) the obtained yield of OTA was much higher in the first case (6.40 mg/l compared to 0.80 mg/l) (Table 2). The obtained results pointed out that aeration influenced the toxin biosynthesis much more than the temperature during the cultivation of *A. ochraceus* E'G in liquid media did.

Much higher amounts of ochratoxin A, between 500 and 300 mg/l, were obtained by fungal isolates representing different sections of the *Aspergillus* genus (Varga et al., 2002). These authors have found the maximum of OTA biosynthesis after 7—10 days of incubation at 30°C in YES liquid medium.

Tab. 3 — Influence of type of the media, temperature and duration of cultivation on ochratoxin A production by *A. ochraceus* E'G

Temper. (°C)	*Days*	*Moist. (%)*	*Medium*	*Yield (mg/kg)*
21—23	21	63.6	rice	96.0
30 ± 1	7	—	corn	n.d.
		—	wheat	20.0
	14	37.8	corn	80.0
		48.8	wheat	240.0
	21	33.0	corn	80.0
		46.6	wheat	800.0
	28	32.0	corn	80.0
		43.0	wheat	800.0

Legend: n.d. — not detected (< 0.004 mg/kg)

The dynamics of OTA production by the isolate *A. ochraceus* E'G was tested during cultivation on three different types of wet cereal kernels (Table 3). The moisture content of corn and wheat grains decreased during prolonged cultivation from 37.8% for corn and 48.8% for wheat, respectively after 14-day cultivation to 32.0% for corn and 43.0% for wheat at the cultivation end (28 days).

Accumulation of the ochratoxin A was much higher in solid substrates then in the liquid media (Table 2 and 3). After three weeks of cultivation of E'G isolate, the similar concentrations of ochratoxin A were recorded on corn kernels and rice grain (80.0 mg/kg and 96.0 mg/kg, respectively) while the highest amounts were found on wheat grain (800.0 mg/kg). Thus, it was observed that the type of cereal grain affected the biosynthetic potential of the fungus, while the cultivation temperature was not so important factor for the toxin production.

Contrary to our findings, the investigations of Sanchis et al. (2006) on OTA production patterns on different substrates, such as YES, barley grains etc. did not show a significant influence of these substrates. On the other hand, these authors found that abiotic factors, such as temperature and water activity, affected significantly toxin biosynthesis. Maximal ochratoxin A accumulation was detected at 25°C and 0.98 a_w on all substrates tested.

The beginning of ochratoxin A production was observed seven days after the cultivation on wheat grain (20.0 mg/kg), and in the case of corn kernel, it was after the prolonged cultivation of *A. ochraceus* E'G for 14 days (80.0 mg/kg). Similar results were obtained by Häggblom (1982) who tested the dynamics of OTA biosynthesis on barley grain by *A. ochraceus* and *Penicillium viridicatum.* This author detected toxin 4 to 6 days after inoculation at 25°C, and observed its maximal accumulation after 28 days (from 7 to 46 mg/kg).

Contrary to wheat substrate, where yield of OTA was increasing until the 21st day of cultivation, the corn substrate did not show any changes in the quantity of the produced toxin from the 14th day of cultivation until the end of the same process (Table 3).

CONCLUSION

Preliminary analysis of *A. ochraceus* E'G potential for the production of ochratoxin A showed that isolate E'G had similar toxicological profile as its parent strain CBS.108.08.

At the end of the cultivation period, in different liquid media, the decrease of initial pH was recorded in all cases, although the most outstanding change occurred after the 10-day stationary cultivation in chamber (27 ± 1°C).

Aeration influenced the toxin biosynthesis much more than the temperature during the cultivation of *A. ochraceus* E'G in liquid media.

The highest yield of OTA (6.4 mg/l) was obtained after submerged cultivation in potato-dextrose broth.

Type of cereal grain used for OTA production showed a significant influence on the dynamic of the process and toxin yields.

After three weeks of cultivation of *A. ochraceus* E'G the similar concentrations of ochratoxin A were recorded on corn kernels and rice grain (80.0 mg/kg and 96.0 mg/kg, respectively) while the highest amounts were found on wheat grain (800.0 mg/kg).

Contrary to wheat substrate where the yield of OTA was increasing until the 21st day of cultivation, the corn substrate did not show any changes in the quantity of the produced toxin from the 14th day of cultivation until the end of the same process.

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IN VITRO БИОСИНТЕЗА ОХРАТОКСИНА А КОД ИЗОЛАТА *ASPERGILLUS OCHRACEUS* E'G

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Резиме

Испитивањем је био обухваћен изолат *Aspergillus ochraceus* E'G изведен из соја *A. ochraceus* CBS 108.08. Прелиминарне анализе присуства охратоксина А (ОТА) су извршене према модификованој методи Filtenborg-a и сар. (1983) на агаризобаним подлогама. Производња токсина је тестирана у следећим течним подлогама: 1) глукоза-пептон-екстракт квасца у бујону (GPY — pH 5,6), 2) кромпир — декстрозном бујону (PDB — pH 6,9), 3) екстракт квасца — сахарозном бујону (YES — pH 6,5) и 4) екстракт квасца-сахарозном бујону са додатком 0,23 mg/l $ZnSO_4$ x 5 H_2O (YES^{Zn} — pH 6,5) у условима стационарне и субмерзне култивације. Динамика биосинтезе охратоксина А праћена је након гајења на природним чврстим супстратима (зрно пиринча, кукуруза и пшенице) током вишенедељне култивације.

Охратоксин А је изолован из течних подлога за култивацију и продукцију применом методе Балзера и сар. (1978) модификоване према Бочаров-Станчић и сар. (2003), док је квантитација ОТА у природним чврстим супстратима извршена према Правилнику о методама узимања узорака и методама физичких, хемијских и микробиолошких анализа сточне хране („Сл. лист СФРЈ", бр. 15/87).

При гајењу изолата *A. ochraceus* E'G у течним подлогама највећи принос ОТА је добијен при коришћењу PDB (6,4 mg/l) и то у условима субмерзне култивације (4 дана, 128 о/мин, 21—23°C). У случају култивације на зрну житарица највећу количину ОТА је изолат *A. ochraceus* E'G биосинтетисао после вишенедељне култивације на зрну пшенице и 30 ± 1°C (800,0 mg/kg).