Inhibition of Ochratoxin A Production of *Aspergillus carbonarius* by Yeast Species

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Abstract

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A total of 21 yeast strains isolated from wine-grapes of Turkey were screened for their ability to inhibit ochratoxin A (OTA) accumulation by *Aspergillus carbonarius* in both yeast extract sucrose (YES) broth and grape juice. In monoculture, *A. carbonarius* produced a mean level of 39.03 µg/l and 21.32 µg/l OTA when grown in YES medium and grape juice, respectively. However, its ability to produce OTA in YES medium was greatly reduced in the presence of yeast strains except for *Candida lusitaniae* E2, *Kloeckera* spp. E4, and *Rhodotorula glutinis* D6. The percentage of inhibition of OTA production in YES medium ranged between 4.67% and 99.87%. Similarly, OTA production was inhibited in grape juice by more than 50% in the presence of yeast strains apart from *Candida famata* E6, *R. glutinis*, *C. famata* O3, *Kloeckera* spp. B3, and *C. lusitaniae* D9. The present study highlights the potential use of yeast isolates in the biocontrol of ochratoxin A-producing *A. carbonarius*.

Keywords: ochratoxin A; *Aspergillus carbonarius*; inhibition; yeast; biocontrol

Ochratoxin A (OTA) is a fungal secondary metabolite that poses a risk to animal and human health due to its carcinogenic, teratogenic, immunotoxic, nephrotoxic, and possibly neurotoxic effects (European Commission 2002a). Ochratoxin A is a well-known nephrotoxic agent and has been associated with fatal human kidney disease referred to as Balkan Endemic Nephropathy and with an increased incidence of tumours of the upper urinary tract (JECFA 2001). In 1993, the International Agency for Research on Cancer (IARC) of WHO classified OTA as a possible human carcinogen (group 2B), based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans (IARC 1993). Recently, increasing interest has been focused on OTA levels in most common foods consumed such as cereals, wine, grape juice, coffee beans, cacao, species, and dried wine fruits (European Commission 2002a). Ochratoxin A is produced by several fungal species in the *Penicillium* and *Aspergillus* genera, primarily *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergilli* of the section *Nigri*, especially *A. carbonarius* (EFSA 2006). While *P. verrucosum* is the main OTA producer in cereals (Lund & Frisvad 2003), *Aspergillus* species such as *A. ochraceus* and *A. carbonarius* are typically associated with coffee, species and grapes (JECFA 2001). Among these species, *A. carbonarius* is considered to be a major source of OTA in grapes and wine (Serra *et al*. 2003; Sage *et al.* 2004).

Because of its harmful effects on human health, several physical and chemical methods have been

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tested for the removal of OTA and other toxins from contaminated commodities. However, the use of many of the available physical and chemical methods including the heat-treatment, irradiation, extraction with sorbents and chemical agents is restricted due to the problems concerning the safety issues and, possible losses in the nutritional quality of the treated commodities (Kabak *et al.* 2006). This has led to the search for alternative strategies such as biological agents. While much work in this area has been performed on aflatoxins (Karunaratne *et al.* 1990; Cotty 1994; Dorner *et al.* 2003; Kabak *et al*. 2009), much less information is available on OTA (Petersson *et al*. 1998; Fuchs *et al.* 2008), deoxynivalenol (Niderkorn *et al.* 2006), fumonisin B_1 (El-Nezami *et al*. 2002) and other toxins. In this respect, we studied the ability of yeasts isolated from wine grapes of Turkey to inhibit OTA production by *A. carbonarius* A2034.

MATERIAL AND METHODS

Reagents and standard. All solvents used for the liquid chromatographic mobile phases were obtained from Merck (Darmstadt, Germany) and were of HPLC grade. In all analytical steps, ultrapure water from the Milli-Q apparatus (Millipore, Bedford, USA) was used. Ochratoxin A was purchased from Sigma-Aldrich (St. Louis, USA) as a crystalline powder form. OCHRAPREP® Immunoaffinity column (IAC) containing specific monoclonal antibodies bound to a solid support material for OTA clean-up were obtained from R-Biopharm Rhone diagnostic (product code: P14B; Glasgow, Scotland). Phosphate-buffered saline (PBS) was prepared by dissolving 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g anyhydrous Na_2HPO_4, and 8.0 g NaCl in 900 ml water. The pH of the PBS buffer was adjusted to 7.4 with 0.1 mol/l HCl or NaOH and the buffer was diluted to 1000 ml.

Grape samples. During 2005 season, grapes were harvested in three important wine-producing regions in Turkey, which belong to central Anatolia, east Anatolia and Aegean region. Six grape varieties were included: Emir, Dimrit, Kalecik Karası (central Anatolia), Bogazkere and Okuzgozu (east Anatolia) and Sultaniye (Aegean region). The bunches were collected in sterile bags, transported to laboratory and stored at 4 ± 1°C until analysis.

Isolation and identification of yeasts from grapes. For each grape variety, a sample using 10 g of berries randomly selected was prepared by transferring them aseptically into 90-ml sterile peptone water (0.1% w/v) and thorough mixed. Each suspension (0.1 ml) was plated over both yeast extract agar and malt extract agar (both Merck, Darmstadt, Germany) media. All plates were incubated for 3 days at 25°C. The strains were purified and maintained in yeast extract tubes under refrigeration, covered with sterile paraffin to reduce their metabolic activity and to avoid agar dehydration.

The isolates were identified using the API 20C test strips (Biomérieux, Craponne, France). The identification with the API 20C system is based on 19 carbohydrate assimilation tests. The experiments were carried out according to the manufacturer's instructions. The inoculation of the tubes was performed by adding a yeast suspension with a turbidity equivalent to a 2 Mac Farland to the dehydrated substrates. The strips were incubated at 30°C in a plastic incubation tray and then read for the presence of the growth (turbidity) at 24 h, 48 h, and 72 hours. The results were transformed into a numerical the profile which was compared with those given in profile list in APILAB identification package program.

Yeast isolates, fungal culture, and growth conditions. Yeast isolates, mainly belonging to *Candida* and *Kloeckera* were activated in yeast extract broth (0.5% yeast extract, 2% glucose) at 25°C for 3 days under aerobic conditions. At the end of the incubation period, the number of yeast cells was enumerated by standard plate count method using yeast extract agar (Merck, Darmstadt, Germany). *A. carbonarius* A2034, an OTA producer was obtained from Instituto di Patologia Vegetale Laboratory, Cattolica del Sacro Cuore University, Piacenza, Italy, and grown on malt extract agar (Merck, Darmstadt, Germany).

Preparation of spore suspension of *A. carbonarius*. After incubation, the spores were harvested using 5 ml of sterile PBS containing 0.05% Tween-80 by brushing the slant surface with a sterile inoculating loop. Conidia were aseptically filtered through sterile cheese cloth to remove mycelial fragments. Both mould spores and yeast cell concentrations were determined using the traditional plate count method and adjusted to 10^6 spores/cells per ml of sterile peptone water (0.1% peptone, w/v).

Effect of yeasts cells on OTA production by** A. carbonarius **in medium. To one ml of each cell suspension (10^6 cells/ml) in a 250 ml Erlenmayer flask containing 50 ml of yeast extract sucrose broth (YES, 2% yeast extract, 15% sucrose, pH 6.5) 10^6 spores/ml of *A. carbonarius* were added. The Erlenmayer flasks were incubated at 30°C for 15 days and then analysed for OTA production. The single culture inoculation assay served as a negative control. All assays were performed in triplicate.

Effect of yeasts cells on OTA production by** A. carbonarius **in grape juice. Fresh grapes (cultivar Sultaniye) were crashed and then filtered through two layers of cheese cloth. The grape juice was pasteurised at 90°C for 5 minutes. The presence of OTA in grape juice used in the analysis was estimated out according to the method reported by Serra *et al.* (2004) with slight modifications. Briefly, grape juice (50 ml) was mixed with 50 ml of methanol and 5 ml of 0.1 mol/l *orto*-phosphoric acid in a blender for 2 minutes. The mixture was filtered through a glass microfibre filter (1.5 µm pore size). An aliquot (12.5 ml) of the filtrate was diluted to 100 ml with PBS, and the diluted extract was passed through the IAC at a flow rate of about 2–3 ml per minute. The column was washed with 20 ml water (2 × 10 ml) and dried under nitrogen. OTA was eluted with 1.6 ml methanol/acetic acid (99:1, v/v), carefully evaporated in the vial to dryness under a stream of nitrogen and redissolved in 400 µl mobile phase. Finally, 20 µl of the aliquot was injected onto the HPLC column.

Both cell and spore suspensions were treated as described earlier, but instead of YES broth, the cell and spore suspensions were inoculated into grape juice-OTA free.

Preparation of standard solution. The stock solution (approx. 500 µg/ml) was prepared by dissolving 1 mg of OTA in 2 ml of toluene/acetic acid (99/1, v/v). The solution was left overnight at room temperature to ensure complete dissolution of the crystalline OTA. 50 µl of OTA standard stock solution was transferred to a 25 ml brown volumetric flask and was quickly diluted with toluene/acetic acid to obtain a working solution, at 1 µg/ml. Standard working solution was stored at –18°C in the dark; the solution was brought to room temperature before use.

Ochratoxin A analysis. The method developed by Petersson *et al.* (1998) was used with some modifications for the present analysis. At the end of the incubation period, the mixture was passed through Whatman No. 4 filter paper. In a test tube, 1 ml of the filtrate was mixed with 1 ml of chloroform and then centrifuged at 3000× g for 10 minutes. After centrifugation, the clear organic phase at the bottom was evaporated under the stream of nitrogen at < 35°C. The residue was dissolved in 1 ml of HPLC mobile phase and stored at 4 ± 1°C until the HPLC analysis. All samples were passed through a 0.45 µm membrane filter prior to injection onto the HPLC column.

The HPLC system (Agilent 1100) consisted of isocratic pump (Agilent G 1310 A9), fluorescence detector (Agilent 1100) and CSI 6150 online vacuum degasser (Cambridge Scientific Instruments, England). Chemstation 3D software (Agilent) was used to control the system and the process signals. The separation was performed on a Silica 5 µm ACE 5 C18, 100 Å, 25 × 4.6 mm column supplied with Advanced Chromatography Technologies (Aberdeen, Scotland). A Rheodyne 7725i stainless steel manual injector (Agilent, Palo Alto, USA) with 20 µl loop was used. Two injections were performed for each sample. A mixture of acetonitrile-water-acetic acid (47:51:2, v/v/v) at 1 ml/min flow-rate was used as the mobile phase. The detection of OTA was carried out using 333 nm and 470 nm as the wavelengths for excitation and emission, respectively. Quantification of OTA was performed by measuring its peak area with the help of the calibration curve calculated from standard solutions. The correlation coefficient was linear (r^2 = 0.99850). The retention time was about 13 min for OTA. The LOD (limit of detection) and LOQ (limit of quantification) of the method were 0.12 ng/ml and 0.40 ng/ml for medium and 0.18 ng/ml and 0.59 ng/ml for grape juice, respectively.

Recovery performance. Yeast extract sucrose broth and grape juice-OTA free were spiked with OTA at the levels of 2 ng/ml, 5 ng/ml and 10 ng/ml. Spiking was carried out in triplicate and a single analysis of the blank sample was performed. The recovery percentage was calculated for both YES broth and grape juice individually. OTA concentrations were determined using the protocol previously described.

RESULTS AND DISCUSSION

Method performance

Table 1 shows the results of the recovery studies. The average OTA recovery for YES broth varied

Table 1. Recovery data for ochratoxin A in YES broth and grape juice samples

Spiked samples	Spiking level (ng/ml)	OTA detected (mean ± SD) (ng/ml)	Recovery (mean ± SD) (%)	RSD (%)
YES broth	2	1.550 ± 0.120	77.48 ± 6.00	7.74
	5	3.826 ± 0.313	76.52. ± 6.24	8.15
	10	7.359 ± 0.685	73.59 ± 6.85	9.30
Grape juice	2	1.494 ± 0.159	74.70 ± 5.30	7.09
	5	3.721 ± 0.312	74.41 ± 6.25	8.40
	10	7.111 ± 1.166	71.11 ± 11.66	16.40

SD – standard deviation; RSD – relative standard deviation

from 73.59% to 77.48% (mean 75.86%), and relative standard deviation (RSD) ranged from 7.74% to 9.3% (mean 8.4%). The recoveries in grape juice were 71.11–74.70% (mean 73.41%) with the 2–10 ng of OTA per ml of sample, and the RSD range for the recoveries was 7.09–16.40 % (mean 10.63%) at these spiking levels. As shown in Table 1, the highest recovery rate of OTA was obtained at the level of 2 ng/ml, instead of that at the level of 10 ng/ml. The recoveries for grape juice obtained with the presented method were lower than those reported by Ng *et al.* (2004), Rosa *et al.* (2004), and Czerwiecki *et al.* (2005) since the analytical procedure was simpler without IAC clean-up prior to HPLC injection. However, the recovery rates of the presented method are acceptable according to the European guidelines for method validation in the European Commission directive 2002/26/EC (European Commission 2002b). In the EU legislation, it is stated that, at < 1 µg/kg and between 1 µg/kg and 10 µg/kg, the recoveries are acceptable in the range of 50–120% and 70–110%, respectively.

Effect of yeast strains on OTA accumulation

In the present study, *A. carbonarius* produced a mean level of 39.03 µg/l OTA when grown alone in YES medium, after 15 days of incubation at 30°C. However, considerably lower amounts of OTA (21.32 µg/l) were produced by *A. carbonarius* in grape juice, under same the conditions. Ochratoxin A production in YES medium was about two fold higher than in grape juice. This result clearly shows that the substrate is one of

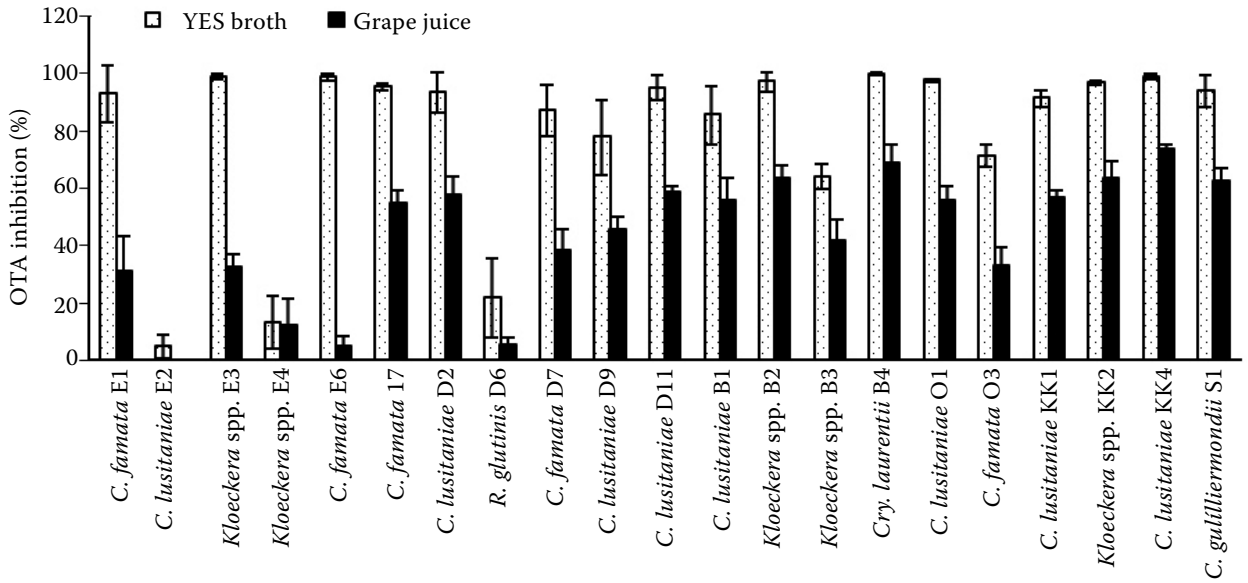

Figure 1. Inhibition of OTA by yeast isolates both on YES broth and grape juice

the important factors in the production of OTA. A previous study by Cabañes *et al.* (2002) observed that *A. carbonarius* isolates produced maximum OTA on unmodified YES agar and Czapek yeast agar after about 14 days. In a recent study, Serra *et al.* (2006) found that the levels of OTA produced by black *Aspergillus* strains on the grape juice medium were lower than those produced on Czapek yeast agar.

The ability of yeasts isolated from wine-grapes of Turkey to inhibit OTA production by *A. carbonarius* were assessed both in YES medium and grape juice. In this study, a total of 21 yeast strains were isolated, being 38% *C. lusitaniae* strains, 24% *Candida famata*, and 24% *Kloeckera* strains. Figure 1 shows the percentage of OTA inhibition in the presence of the yeast isolates in both YES medium and grape juice. The production of OTA in YES medium was greatly reduced in the presence of yeast strains except for *C. lusitaniae* E2, *Kloeckera* spp. E4 and *Rhodotorula glutinis* D6 strains. The percentage of the inhibition of OTA production in YES medium ranged between 4.67% and 99.87%. HPLC chromatograms of YES broth after the treatment with *Kloeckera* spp. E3 and untreated control are shown in Figure 2. On the other hand, among the yeast isolates only *C. lusitaniae* E2 could not inhibit OTA production in grape juice. The other isolates were able to prevent OTA production by *A. carbonarius* the percentage of inhibition ranging from 5% to 73.5%, in grape juice.

Yeasts are predominant microorganisms in the natural flora on the surface of fresh grapes and are significant in winemaking since they carry out the alcoholic fermentation (Moreno-Arribas & Polo 2005). Several antagonistic yeasts have been reported to reduce the growth of ochratoxigenic fungi as well as OTA production. *Pichia anomala* and *Saccharomyces ceevisiae* are known to be effective in reducing OTA accumulation *in vitro* in two isolates of *P. verrucosum*, while *Pichia anomala* can reduce OTA levels synthesised by *P. verrucosum* from 100 000 ng/g to < 10 ng/g level in wheat at 25°C after 21 days (Petersson *et al.* 1998). In addition, *Kluyveromyces* isolates, Y_{14}, Y_{16}, Y_{22}, and Y_{25} have also been demonstrated to inhibit both the growth and AFB_1 accumulation in *Aspergillus* section Flavi (La Penna & Etcheverry 2006). Interestingly, yeast species isolated from grape berries, namely *Issatchenkia orientalis*, *Metschnikowia pulcherrima*, *Issatchenkia terricola*, and *Candida incommunis* have been reported to reduce colonisation of grape berries by *A. carbonarius* and *A. niger*, the main species responsible for the accumulation of OTA in grapes (Bleve *et al.* 2006)

Microbial interactions can both stimulate and inhibit mycotoxin accumulation depending on the species and environmental factors. In contrast to the results of this study, Kabak and Var (2004) reported that AFB_1 production was stimulated by interaction with *Lactobacillus* and *Bifidobacterium* strains. However, there are few data about the mechanisms of antifungal/antimycotoxigenic effects of antagonistic microorganisms. It has been suggested that extracellular metabolites produced in the growth medium may play an important role in the inhibitory activity of microorganisms. Kimura and Hirano (1988) found that antiaflatoxigenic activity of *Bacillus subtilis* was due to inhibitory compounds other than organic acids produced in the growth medium.

In conclusion, the effect of yeast isolates on the production of OTA by *A. carbonarius* varied and was affected by the substrate. In further studies,

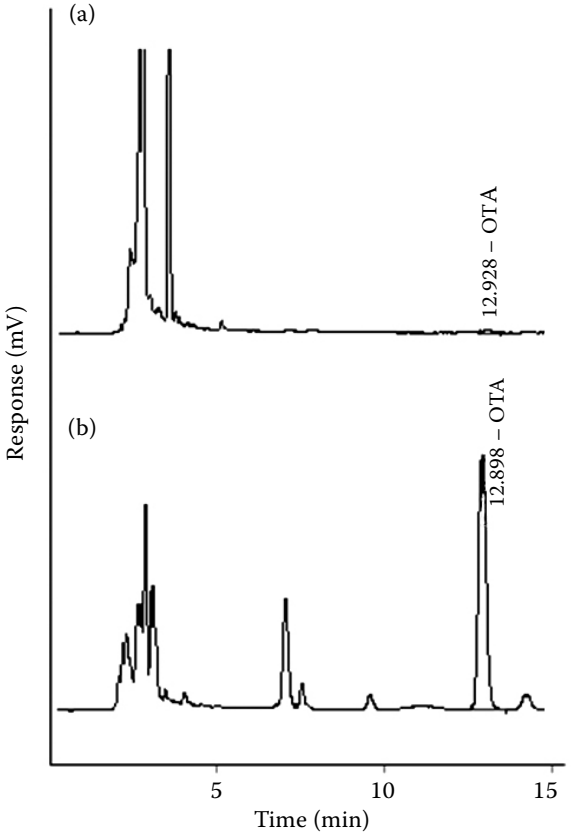

Figure 2. HPLC chromatograms of YES broth after treatment with *Kloeckera* spp. E3 (a) and untreated control (b)

the potential use of yeasts to degrade OTA in fermentation processes should be investigated. More work is needed to determine antifungal/anti-mycotoxigenic mechanisms involved.

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