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Mechanisms involved in reduction of ochratoxin A produced by *Aspergillus westerdijkiae* using *Debaryomyces hansenii* CYC 1244

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ABSTRACT

Aspergillus westerdijkiae is one of the most relevant ochratoxin A (OTA) producing species within the Section Circumdati contaminating a number of agroproducts. The yeast *Debaryomyces hansenii* CYC 1244 was previously reported to be able to reduce growth and extracellular OTA produced by *A. westerdijkiae*. In this work, we examined several mechanisms possibly involved in this OTA reduction in in vitro experiments. OTA biosynthesis was evaluated by quantitation of expression levels of *pks* (polyketide synthase) and *p450-B03* (cytochrome p450 monooxygenase) genes using newly developed and specific real time RT-PCR protocols. Both genes showed significant lower levels in presence of *D. hansenii* CYC 1244 suggesting an effect on regulation of OTA biosynthesis at transcriptional level. High levels of removal of extracellular OTA were observed by adsorption to yeast cell walls, particularly at low pH (98% at pH 3). On the contrary, no evidences were obtained of absorption of OTA into yeast cells or the production of constitutively expressed enzymes that degrade OTA by *D. hansenii* CYC 1244. These results described the potential of this yeast strain as a safe and efficient biocontrol agent to decrease OTA in *A. westerdijkiae* and two important mechanisms involved which may permit its application at different points of the food chain.

1. Introduction

Ochratoxin A (OTA) is one of the most important mycotoxins due to its toxic properties towards both animals and humans (Pfohl-Leszkowicz and Manderville, 2007). Since 2006, maximum OTA levels in a large number of raw products are legislated by the European Union (Commission Regulation, 2006). Traditionally, *Aspergillus ochraceus* was considered the main source of OTA in warm climates and *Penicillium verrucosum* the most important producer in cold temperate regions (Mantle, 2002). However, the development of molecular techniques supposed the description of new species that have replaced these as the main OTA-producing species. Frisvad et al. (2004) described new species in *Aspergillus* section Circumdati and our group has demonstrated, in a recent work, that *Aspergillus westerdijkiae* is more relevant as OTA producer than *A. ochraceus* (Gil-Serna et al., 2011). Up to date, *A. westerdijkiae* presence has been reported so far in coffee (Leong et al., 2007; Noonim et al., 2008), grapes (Diaz et al., 2009) and cocoa beans (Coppeti et al., 2010) and, in our group, different isolates from several sources such as paprika, cereals and nuts have been re-classified as *A. westerdijkiae* (Gil-Serna et al., 2009b, 2011).

Control of ochratoxigenic fungi in fields or during storage is the best strategy to prevent OTA contamination of food products (Amézqueta et al., 2009; Kabak and Dobson, 2009). Chemical products have been commonly used to reduce fungal proliferation and mycotoxin production in fields. However, nowadays a strict legislation about their use has been established in the European Union and maximum residue levels of these compounds have been regulated in many products (Commission Regulation, 2008) due to the increasing number of resistant fungal strains (Hollomon et al., 1997) and the impact of fungicides on environment and human health (De Costa and Bezerra, 2009). Therefore, alternative methods are necessary to substitute or complement treatments with fungicides to control fungi in fields or storage places.

Biological control using antagonistic microorganisms has been proposed for a long time as a good option to control plant pathogens (Wilson and Wisniewski, 1989). One of the advantages of biocontrol is that it could be use together with fungicides reducing their levels in order to decrease fungal growth (Spadaro and Gullino, 2004).

Yeasts are considered one of the most potent biocontrol agents due to their biology and non toxic properties (Pimenta et al., 2009) and one strain of *Candida sake* has even been patented in a European context for its use against fungal pathogens in apples and pears (Viñas et al., 1998).

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The mechanism most probably involved in biocontrol of fungi by yeast is competition (Spadaro and Gullino, 2004; Zhou et al., 2007) although parasitism and production of fungal growth inhibiting compounds have been also described (El-Tarabily and Sivasithamparam, 2006; Pimenta et al., 2009). Nowadays, several yeast species included in different genera are considered biocontrol agents towards ochratoxigenic *Aspergillus* fungi (Bleve et al., 2006; Dimakopoulou et al., 2008; Masoud and Kaltoft, 2006; Zahavi et al., 2000).

When prevention of OTA production cannot be controlled, detoxification of foodstuffs is essential to avoid the toxin entering the food chain. There are different detoxification methods but their use is limited because they usually have also negative effects for human health and environment or alter nutritional and organoleptic properties of products (Amézqueta et al., 2009; Kabak and Dobson, 2009). Because of this, chemical treatments are not allowed in the European Union (Commission Regulation, 2006).

Debaryomyces hansenii is widely used in biotechnological processes because of its characteristic metabolic properties (Breuer and Harms, 2006), and several strains are considered as good biocontrol agents against fungal pathogens included in the genera *Penicillium* in different fruits like apples, grapes or citrus (Chalutz and Wilson, 1990; Droby et al., 1989; Hernández-Montiel et al., 2010). Recently, Liu and Tsao (2009) described also the application of *D. hansenii* to control dairy molds in yogurt and cheese. In previous studies carried out in our group, we have demonstrated that *D. hansenii* CYC 1244 is not only able to reduce significantly *A. westerdijkiae* growth in agar plates, but also OTA decreased or was even not detected in the medium when the yeast is present (Gil-Serna et al., 2009a).

Several authors have studied the mechanisms involved in OTA reduction by yeasts. Some species are capable to produce carboxypeptidases which transform OTA to its less toxic derivative OTA-α (Abrunhosa et al., 2010; Molnar et al., 2004) and others are able to degrade the toxin by unknown ways (Angioni et al., 2007; Péteri et al., 2007). Adsorption of OTA to *Saccharomyces cerevisiae* cell wall during fermentation of must and wines has been previously reported in many works (Bejaoui et al., 2004; Caridi et al., 2006; Cecchini et al., 2006; Garcia Moruno et al., 2005). Moreover, this mechanism is also involved in OTA removal by different genera such as *Phaffia*, *Candida*, *Kloeckera* and *Rhodotorula* (Péteri et al., 2007; Var et al., 2009). Recently, Meca et al. (2010) have reported absorption into the cell in *S. cerevisiae* as a new mechanism for OTA detoxification.

Description of two genes, *pks* and *p450-B03*, involved in OTA biosynthesis in *A. westerdijkiae* (formerly *A. ochraceus*) has been recently reported (O'Callaghan et al., 2003, 2006). The genes code for a polyketide synthase and a cytochrome p450 monooxygenase, respectively. Additionally, in that work, the authors demonstrated that the regulation of OTA production occurs at transcriptional level and the expression of these genes could be correlated with extracellular OTA levels detected.

The aim of this work was to examine the mechanism or mechanisms involved in the reduction of OTA extracellular amount by *D. hansenii* CYC 1244. Adsorption to wall, absorption into the cell and degradation were evaluated. Moreover, reduction of OTA biosynthesis by *A. westerdijkiae* was determined by analyzing the expression of two biosynthetic genes (*pks* and *p450-B03*). Toxin concentration was measured by HPLC.

2. Materials and methods

2.1. Fungal isolates and culture conditions

Two OTA-producing strains of *A. westerdijkiae* were tested in this study, the type species CECT 2948 and the strain AOPD16-1 isolated from paprika and kindly provided by Dr Ramos (University of Lleida, Spain). The correct classification of the isolates was tested by specific PCR protocols according to Gil-Serna et al. (2009b). They were maintained by regular subculturing during the experiment on Potato Dextrose Agar (PDA, Pronadisa, Madrid, Spain) at 25 ± 1 °C for 4–5 days and then stored at 4 °C until required and as spore suspension in 15% glycerol at −80 °C.

The biocontrol agent *D. hansenii* CYC 1244 was used on the basis of previous studies carried out by our group (Gil-Serna et al., 2009a). It was maintained by regular subculturing during the experiment on YMA (Wickerman, 1951) at 27 ± 1 °C for 24 h and then stored in 15% glycerol at −80 °C until required.

2.2. OTA quantification

OTA was measured in the extracellular extracts by High Performance Liquid Chromatography (HPLC) on a reverse phase C18 column (Tracer Extrasil ODS2; 5 μm, 4.6 mm × 250 mm; Teknokroma, Barcelona, Spain) at 45 °C in a Perkin Elmer Series 200 HPLC system coupled with a fluorescence detector (Perkin Elmer, Massachusetts, USA) at excitation and emission wavelengths of 330 and 470 nm respectively. The mobile phase contained monopotassium phosphate 4 mM pH 2.5 and methanol (33:67) and the flow rate was 1 ml/min. OTA was eluted and quantified by comparison with a calibration curve generated from OTA standards (OEKANAL®, Sigma-Aldrich, Steinheim, Germany).

2.3. Mechanism 1: Effects on OTA biosynthesis

2.3.1. Culture conditions

An overnight culture of *D. hansenii* CYC 1244 in YMB medium was harvested by centrifugation at 13,000 rpm for 5 min and resuspended in saline solution. Cell concentration was measured by microscopy using a Thoma counting chamber and the suspension was diluted to a final concentration 5×10^4 cells/ml. One milliliter of this suspension was mixed with 25 ml of melted CYA medium containing 45.4 g/l Czapek-Dox Modified Agar (Pronadisa, Madrid, Spain) and 5 g/l yeast extract (Pronadisa, Madrid, Spain). Fungal conidium suspensions were prepared from a sporulating culture (7 day-old) on Czapek Agar and filtered through Whatman N°1 paper. Concentrations were measured by microscopy as described above and the suspensions were diluted up to 10^7 spores/ml. After solidification of the CYA plates inoculated with *D. hansenii*, sterile cellophane membranes (P400, Cannings Ltd, United Kingdom) were laid on the plates to enable removal of the mycelia for gene expression analysis. Spots of 1.5 μl of *A. westerdijkiae* spore suspensions were placed on each plate. Control CYA plates without *D. hansenii* were also inoculated with the spore suspensions. All plates were cultured between 3 and 7 days at 28 °C. Each day fungal growth was determined by measuring the colony diameter and OTA was extracted by a method described elsewhere (Bragulat et al., 2001). Mycelia were removed and kept at −80 °C until RNA isolation. All assays in this experiment were carried out in duplicate.

2.3.2. RNA isolation and cDNA synthesis

Fungal mycelia were frozen with liquid nitrogen and grinded using a mortar and a pestle. RNeasy Plant Mini Kit (QIAgen, Valencia, Spain) was used following manufacturer's instruction and isolated RNA was treated with DNase I (QIAgen, Valencia, Spain). RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, USA).

cDNA was prepared starting from 1 μg of isolated RNA using a reverse-transcriptase enzyme (Roche, Spain) primed using random hexamers (Promega, Spain).

2.3.3. Real-time PCR analysis of gene expression

The expression of a polyketide synthase (*pks*) and a cytochrome p450 monooxygenase (*p450-B03*) genes was studied. Primers sets were designed on the basis of the sequence of these genes described previously by O'Callaghan et al. (2003, 2006) and the sequences of *A. westerdijkiae* ß-tubulin gene (*ßtub*) were previously obtained in our

group (Gil-Serna et al., 2009b). The primer's sequences used in this study are shown in Table 1.

Real-time PCR assays were performed and monitored in an ABI PRISM 7900HT system (Applied Biosystems, Spain) in the Genomic Unit of the University Complutense of Madrid. The final reaction volume (10 μl) contained: 5 μl SYBR® Green PCR Master Mix (Applied Biosystems, Spain), 0.6 μl forward primer 5 μM, 0.6 μl reverse primer 5 μM, 2.5 μl cDNA template and 1.3 μl molecular biology water (MO-BIO, USA). The real time PCR assays were carried out using a standard program: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were carried out by duplicate.

Gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *ßtub* expression was used as endogenous control and the calibrator sample corresponded to the value of the expression of untreated control after 4 days of incubation. A standard curve was generated for all primer pairs to calculate amplification efficiency starting from ten-fold serial dilution of DNA from CECT 2948 strain (from 50 to 5×10^{-3} ng/μl). The relative quantification method only could be applied if differences in amplification efficiencies between the constitutive gene and target genes are less than 10% (Schmittgen and Livak, 2008).

2.4. Mechanism 2: Adsorption to cell wall and/or absorption by yeasts

2.4.1. Preparation of OTA extract

A. westerdijkiae CECT 2948 was cultured in a 1 l Erlenmeyer flask with 250 ml of YES broth (Yeast Extract Sucrose) at 28 ± 1 °C on orbital shaker (120 rpm) for 7 days. Mycelium was removed by filtration using Whatman paper N°1 and the liquid was filtered though a 0.22 μm filter (Sartorius, Spain). The filtered liquid corresponded to the OTA extracts used in subsequent experiments.

2.4.2. Yeast preparation

D. hansenii CYC 1244 suspensions were prepared as described above to final concentrations 10^4 and 10^7 cell/ml. These resulting suspensions constituted the viable cells. Half of the suspensions were autoclaved (121 °C, 20 min) and constituted the heat-killed yeasts. Cells in the extracts were harvested by filtration and resuspended in the same volume of phosphate buffer pH 3, 5 and 7.

2.4.3. Adsorption/absorption assays

Viable cells (800 μl) in all conditions (pH and cell concentration) were mixed with the same amount of OTA extract. These mixes were incubated for 5, 15, 30 min and 1, 3, 9, 24 h at 28 °C with strong shaking (300 rpm). This protocol was carried out with heat-killed yeast too with incubation during 5, 10, 15, 30 min and 1, 3 h and the same conditions.

After incubation, the mixes were filtered through syringe filters (0.22 μm) and OTA was extracted with Ochraprep® Immunoaffinity columns (R-Biopharm, Madrid, Spain) and measured by HPLC as described before. These methods were also used to quantify OTA in the original extract.

Table 1
Real time PCR primers used in this study. The amplification efficiency corresponded to the values calculated from the standard curves generated as described in materials and method.

Gene	Primers	Sequence	Efficiency
ßtub	BTUBWESTF	5′ CTTCCGGTGGCAAGTATGTTC 3′	95%
	BTUBWESTR	5′ CATGGTACCGGGCTCAAGAT 3′	
pks	PKSWESTF	5′ CCCAGCGATAACGCACTGA 3′	94%
	PKSWESTR	5′ ATGGCGGTGCGGAGAGT 3′	
p450-B03	P450WESTF	5′ TTTGGCATTGCGGAACTTTAC 3′	92%
	P450WESTR	5′ CATGTCAAACGTGCGGAAGA 3′	

2.5. Mechanism 3: Degradation of OTA by extrolites produced by *D. hansenii* CYC 1244

D. hansenii CYC 1244 was cultured on YMB medium (50 ml) during 72 h at 27 °C in an orbital shaker (120 rpm). Subsequently, they were filtered twice through 0.22 μm filters to ensure complete removal of cells. Ten milliliter of the filtered medium was mixed with the OTA extract prepared as described above. The mixtures were maintained for 24 and 48 h before the extraction with Ochraprep® Inmunoaffinity columns. A control assay with 10 ml of non inoculated media was carried out to compare the results.

2.6. Statistical analysis

After checking normality and homoscedasticity of the data, a two ways ANOVA was carried out to compare the effects of the different parameters on the absorption/adsorption tests performed. In the case of degradation tests, where only two cases were compared (control and treated), U-Mann Whitney analysis were performed. In all cases, statistical significance was established as $p \leq 0.05$.

3. Results

3.1. Mechanism 1: Effects on OTA biosynthesis

The amplification efficiencies calculated from the slope of the standard curves generated with all the primer sets designed are shown in Table 1. The highest difference value between the amplification efficiency of the constitutive gene and the other two target genes was 3%, therefore the $2^{-\Delta\Delta CT}$ method was applied.

The results of gene expression analysis for *pks* and *p450-B03* genes for both strains are shown in Fig. 1. Both genes were expressed with similar profiles in control plates either in CECT 2948 or AOPD16-1 strains with a maximum after 4 days of incubation, then a decrease until day 6 and finally a new increase of expression at the end of the experiment. However, expression levels of genes involved in OTA biosynthesis in each strain was differently affected by the presence of *D. hansenii* CYC 1244, although a significant reduction was found in both cases. CECT 2948 strain had a maximum of expression at day 6 whereas AOPD16-1 showed higher expression values at the first day of the assay for both, *pks* and *p450-B03* genes. Comparing the maximum of expression in each case, CECT 2948 showed a reduction of *pks* and *p450-B03* expression of 59% and 46%, respectively. AOPD16-1 strain showed a reduction of 64% for *pks* and 62% in the case of *p450-B03*.

Additionally, results of OTA concentration in CYA plates are shown in Fig. 1. In all cases, a marked reduction of toxin amount was found when the fungi were co-cultured with *D. hansenii* CYC 1244.

3.2. Mechanism 2: Adsorption to cell wall and/or absorption by yeasts

Fig. 2 shows the effect of pH in OTA removal by *D. hansenii* CYC 1244 in the extracts. The highest reduction was obtained at pH 3; after 5 min of incubation more than the 98% of initial OTA amount was removed. However, at pH 5 and pH 7, a lower amount of initial OTA was removed (63% and 65%, respectively) after this short incubation. In these latter cases, it is important to remark the high variability found, showing OTA levels which varied markedly along time.

The state of cells (living or death) did not affect OTA removal. Statistically significant differences were not found in OTA removal carried out by heat-killed cells or viable cells. Moreover, there were not differences between the results using both cell concentrations.

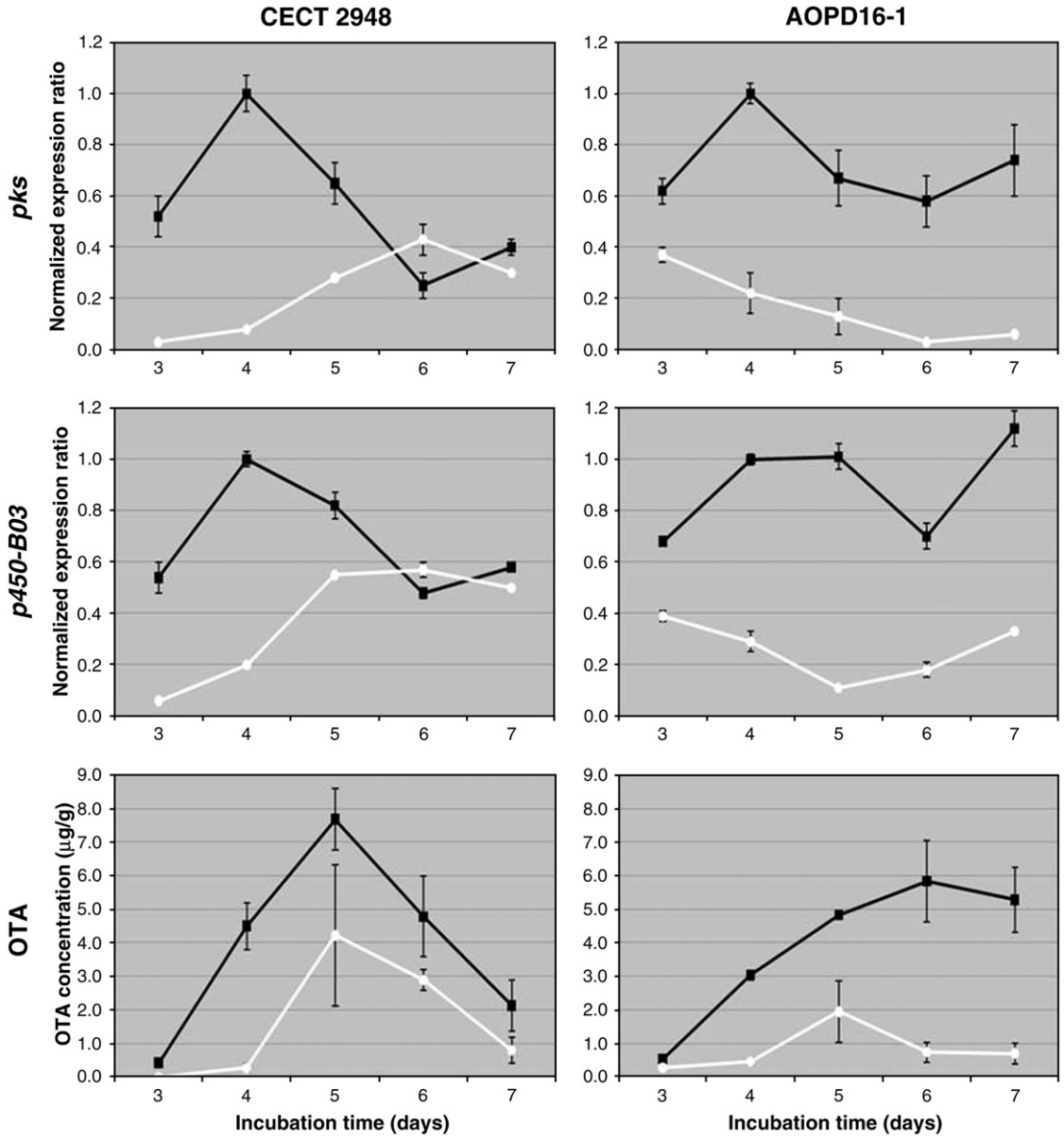

Fig. 1. Normalized expression ratio of *pks* and *p450-B03* genes in both strains tested and OTA concentration in CYA medium. Black lines correspond with non-treated assays (control) and white lines are the results of the two *A. westerdijkiae* strains expression rates in plates with *D. hansenii* CYC 1244. Results are represented in relation to control after 4 days of incubation. Error bars denote the standard error of the mean of two replicates from independent cultures.

3.3. Mechanism 3: Degradation of OTA by extrolites produced by D. hansenii CYC 1244

Table 2 shows OTA concentration in the mixes with YMB medium where *D. hansenii* had been previously grown after 24 and 48 h of incubation. OTA levels in the extract were not modified by the presence of extrolites produced by *D. hansenii* CYC 1244 at any incubation time. Statistically significant differences were not found among OTA concentrations in any case.

4. Discussion

OTA is a highly stable compound and usually resists food processing technologies. No efficient decontamination methods are currently available without varying product properties; therefore prevention remains the most important strategy to avoid the toxin entering the food chain (Amézqueta et al., 2009).

In a previous report, we described the reduction of extracellular OTA in cultures of *A. westerdijkiae* with *D. hansenii* CYC 1244 (Gil-Serna et al., 2009a). In this work, we have confirmed the ability of this yeast to reduce OTA and examined different mechanisms possibly involved in the reduction of OTA levels by *D. hansenii* CYC 1244. We developed a real-time PCR protocol based on the *pks* and *p450-B03* genes to evaluate their expression in two strains of *A. westerdijkiae* in response to *D. hansenii* CYC 1244. The results showed that both *A. westerdijkiae* strains (CECT 2948 and AOPD16-1) showed a reduction in the expression of OTA biosynthetic genes when they were co-cultured with *D. hansenii* CYC 1244. The response was slightly different depending on the fungal strain considered but at least a reduction of 59% for *pks* and 46% for *p450-B03* was observed. Hence, this reduction in the expression of genes involved in OTA biosynthesis would indicate that *D. hansenii* CYC 1244 influences repression of OTA biosynthetic genes, resulting in lower levels of OTA production in *A. westerdijkiae*. This effect might be produced by the blockage of any step in the biosynthetic pathway as

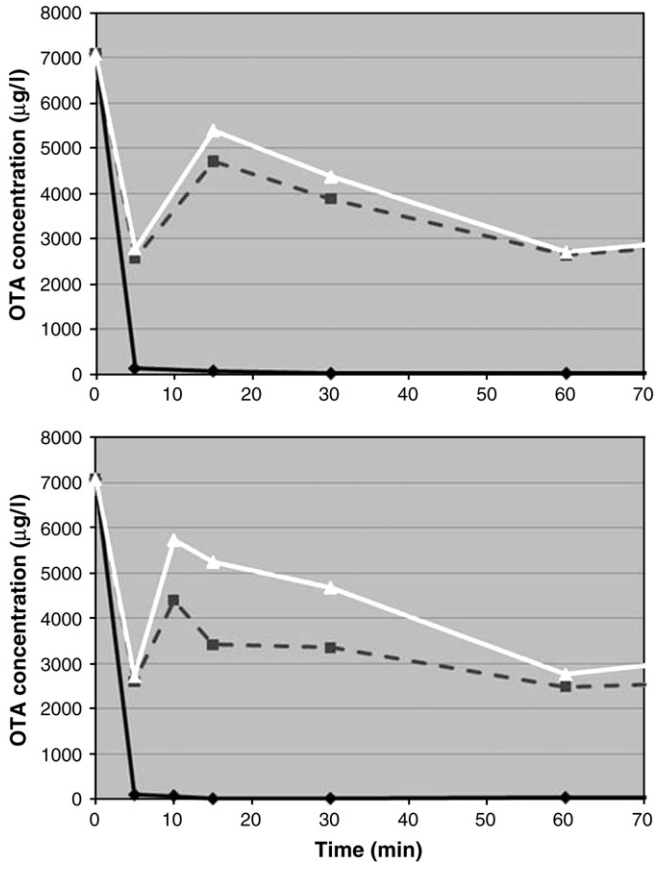

Fig. 2. Effects of pH on OTA removal in 10^4 cells/ml suspensions of viable cells (above) or heat-killed cells (below) of *D. hansenii* CYC 1244. The graphs show the evolution of OTA concentration in the extracts at pH 3 (black line), pH 5 (discontinuous gray line) and pH 7 (white line) up to 70 min of incubation. The concentration of OTA was stable along the rest of the experiment.

Hua et al. (1999) reported in *A. flavus* and its reduced aflatoxin production attributed to saprophytic yeast biocontrol.

Two mechanisms for OTA reduction when co-incubated with yeast cells were studied, namely, OTA adsorption by yeast cell wall or OTA absorption into the cell. The results obtained indicated that OTA adsorption seems to be a more likely mechanism contributing to the observed OTA reduction in comparison with absorption. pH was the most important factor influencing OTA removal by yeast cells, either viable or heat-killed. Adsorption might be produced by a direct linkage of some cell wall molecules and OTA; therefore, ionization state produced by low pH would improve toxin removal by cells. According to this hypothesis, higher pH values (5 and 7) would produce a great instability in the association between toxin and cell wall reducing the ability of cell wall molecules to link OTA and to remove it from the culture.

However, no differences in the reduction of OTA content were found between viable and heat-killed cells. The absorption mechanism would mean internalizing OTA, requiring a metabolically active process which dead cells could not carry out. Furthermore, only five minutes were necessary to reduce the majority of OTA and this discards absorption by *D. hansenii* CYC 1244 because this is a slow process and would have needed more time.

Table 2
OTA concentration in the control toxin extract and after 24 and 48 h of incubation with the YMB culture extract of *D. hansenii* CYC 1244. Results are indicated as mean ± standard deviation of both replicates.

	OTA concentration (μg/L)
Control	6207.9 ± 25.1
24 h	6480.8 ± 17.5
48 h	6543.0 ± 61.7

Although OTA degradation by yeast has been reported in several studies (Angioni et al., 2007; Molnar et al., 2004; Péteri et al., 2007), in this work, we found no evidence of degradation caused by constitutively expressed extrolites produced by *D. hansenii* CYC 1244. No differences were found between control mixes and cell-free cultures of *D. hansenii* and OTA extracts containing the extrolites produced by the yeast, even after 48 h.

Biological methods to control fungal proliferation or to detoxify contaminated products are considered good alternatives to fungicides and chemical processes to prevent or reduced OTA in foodstuffs (Amézqueta et al., 2009). *D. hansenii* is an important biotechnological agent and it is widely used in several processes and applied research (Breuer and Harms, 2006). Nowadays, this species is considered a non-pathogenic microorganism because its opportunistic human infections have been described very rarely, and some authors support the fact that there are very frequently co-occurring fungal pathogens morphologically similar to this species which often results in misidentification (Desnos-Ollivier et al., 2008).

Spreading yeasts with biocontrol properties against *Aspergillus carbonarius* using a spray in field has been demonstrated as efficient as fungicides to control fungal growth (Dimakopoulou et al., 2008). *D. hansenii* CYC 1244 might be a good candidate as biocontrol agent against *A. westerdijkiae* because it seemed not only affect fungal growth as previous reports described (Gil-Serna et al., 2009a) but also its presence reduced OTA biosynthesis in the fungus. We have tested the ability of *D. hansenii* to control *A. westerdijkiae* in artificially inoculated grapes and fungal growth was reduced for an average of 50% (data not shown), therefore this biocontrol agent could be applied to control this fungus in this substrate frequently contaminated by *A. westerdijkiae*.

Moreover, *D. hansenii* CYC 1244 could be used to detoxify foodstuffs since it proved to be capable to remove OTA by adsorption to its wall. The application of this yeast, even dead cells, could be appropriate to remove OTA from beverages often contaminated by the toxin such as musts, wines or juices.

In conclusion, two mechanisms involved in OTA removal by *D. hansenii* CYC 1244 were described. Reduction of toxin biosynthesis by *A. westerdijkiae* and adsorption to yeast cell wall can be proposed as the main mechanisms involved, whereas absorption into the cell and degradation by constitutive enzymes could be discarded.

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