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Effect of pH on the ultrastructure of the conidial wall of *Aspergillus niger* aggregate conidia and its relationship to zearalenone adsorption

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RESEARCH ARTICLE

Abstract

The purpose of the present study was to evaluate the effect of pH on the ultrastructure of the conidial wall of dead conidia harvested from the non-toxicogenic *Aspergillus niger* aggregate strain RC084 and its relationship to zearalenone (ZEA) adsorption capacity. Moreover, mathematical models were applied to explain the interaction between conidia and ZEA absorbance. A ZEA adsorption test was performed using a concentration of 1×10^7 dead conidia/ml at pH 2 and 6 at 37 °C for 30 min. Unbound ZEA was quantified by high-pressure liquid chromatography. The ZEA adsorption was strongly dependent on the pH of the medium; the highest values were 2.2×10^{-6} µg ZEA/conidia at pH 6. Isotherms representing the amount of bound ZEA as a function of ZEA concentration in equilibrium after adsorption have typical S- or L-shapes. The experimental data could be fitted to the Frumkin-Fowler-Guggenheim (FFG) and Hill theoretical models. The ultrastructure of the conidial wall was studied by infrared spectroscopy (IR) and transmission electron microscopy (TEM). The representative IR spectra showed that pH did not produce significant changes in the different chemical groups. However, ultrastructure studies by TEM detected considerable changes in the organisation of the conidial wall. This is the first study showing that the loss of the outermost electron dense layer, responsible for the ornamentations on the conidial surface, at pH 6 potentiates ZEA adsorption.

Keywords: adsorption, dead conidia, isotherms, pH effect, ultrastructure, zearalenone

1. Introduction

Zearalenone (ZEA) is a nonsteroidal mycotoxin produced by several *Fusarium* species (Figure 1). Major effects of ZEA can be found in farm animals, especially in pigs, in the urogenital system. They are characterised by oestrogenism, leading to atrophy of testicles and ovaries, enlargement of mammary glands, abortion and in severe cases, impaired reproductive cycle, conception, ovulation, foetal implantation and development, ultimately leading to infertility (Gremmels and Malekinejad, 2007). Due to the importance of ZEA in animal production, it is extremely important to find adsorbents able of capturing this toxin, which would reduce the deleterious effects of mycotoxicoses

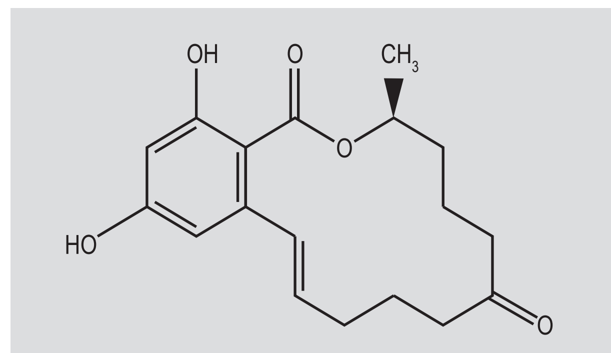

Figure 1. Molecular structure of zearalenone.

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on productive parameters and the transfer of ZEA to animal-derived food intended for human consumption.

One of the most effective methods for controlling mycotoxin exposure in animal husbandry is based on the use of specific materials that adsorb mycotoxins, thus limiting their bioavailability in the body (Gremmels and Malekinejad, 2007). Microbial biomass is particularly useful for sequestering toxins through a passive process known as biosorption. Conidia of black aspergilli are potentially useful as adsorbents in reducing the toxic effects of mycotoxins in animal production. Bejaoui *et al.* (2005) evaluated the capacity of live conidia of black *Aspergillus* isolates to adsorb ochratoxin A (OTA) in both synthetic and natural red grape juices. The physicochemical properties of the conidial surface makes them potential candidates as mycotoxins adsorbents. The hydrophobic nature of the conidial walls has been extensively used by fungi as a virulence factor for humans, animals and plants (Wasylanka and Moore, 2000). These properties may also be involved in the attachment of different mycotoxins found in feeds used for livestock production. Taking the hydrophobic nature of ZEA into account, conidia could be good candidates for use in the prevention of ZEA-associated problems. As there is little information on this subject, the purpose of the present study was to evaluate the effect of pH on the ultrastructure of the walls of dead conidia harvested from a non-toxicogenic *Aspergillus niger* aggregate strain, as well as its relationship to ZEA adsorption capacity. Several mathematical models have been published to describe the equilibrium between adsorbed and free toxin in solutions containing cell walls of yeasts (Yiannikouris *et al.*, 2003). In our study, similar mathematical models were used to interpret the data on toxin adsorption by walls of dead *Aspergillus* conidia.

2. Materials and methods

Aspergillus niger strain

A. niger aggregate strain RC084 was used in this study. It was originally isolated from pig feed and sub-cultured on malt extract agar (MEA; Samson *et al.*, 2014). The strain was identified to species level using the morphological criteria of Klich (2002) and Pitt and Hocking (2009) for macro- and microscopic observation. The strain was deposited at the National University of Río Cuarto, Córdoba, Argentina (RC) collection centre as *Aspergillus niger* aggregate RC084.

The inability of *A. niger* RC084 to produce OTA was confirmed using the methodology described by Bragulat *et al.* (2001). Ochratoxin was quantified on the basis of HPLC fluorometric response compared with an OTA standard (Sigma-Aldrich, St. Louis, MO, USA; purity >99%). The limit of detection of this method was 0.1 ng/g.

Preparation of conidial suspensions

The strain was grown in Czapek Yeast Autolysate agar (CYA; Samson *et al.*, 2014) at 28 °C for 7 days in the dark. After the incubation period, a loop of conidia was harvested from the colony surface and placed in a tube. Three consecutive washes with distilled water were carried out to remove any impurities and mycelium. Dead conidia were obtained by boiling living spores in distilled water for 15 min, followed by centrifugation at 5,000 rpm and discarding the supernatant. A suspension of dead conidia was inoculated on MEA, incubated at 25 °C for 7 days and served as negative control. Conidia were resuspended in solutions at pH 2 (50 ml of potassium chloride 0.2 M and 13 ml of hydrochloric acid 0.2 M) and pH 6 (100 ml of potassium dihydrogen phosphate 0.1 M and 11.2 ml of sodium hydroxide 0.1 M) for subsequent use in the adsorption test. The pH was confirmed with a Model 250A pH meter (Orion Research Inc. Boston, MA, USA) and the corresponding pH was adjusted using hydrochloric acid 0.2 M or sodium hydroxide 0.1 M solutions. The conidial concentrations were counted using a Neubauer chamber and set to 1×10^7 dead conidia/ml.

Adsorption capacity *in vitro*

The adsorption test was performed using a concentration of 1×10^7 dead conidia/ml at pH 2 and pH 6, as proposed by Bejaoui *et al.* (2005). An aliquot of 500 µl dead conidia suspension was added to an Eppendorf vial containing 500 µl of 0.5, 5, 10, 20 and 50 µg/ml ZEA. Each vial was placed into a Labor 2K15 centrifuge (Sigma-Aldrich) at 37 °C with mechanical stirring for 30 min. Eppendorf vials were then centrifuged for 10 min at 14,000 rpm. The supernatants were taken and evaporated to dryness under a gentle stream of nitrogen gas. Two replicates of each adsorption test and controls were performed. The adsorbed amount of ZEA was determined by calculating the difference between the initial amount of ZEA in the solution and the amount left after adsorption. ZEA concentration in the extracts were quantified by HPLC.

Detection and quantification of zearalenone

The method used to quantify ZEA has been described by Cerveró *et al.* (2007). Extracts were resuspended in mobile phase methanol:water (70:30, v/v) and injected into the HPLC. Detection and quantification of ZEA was performed using a fluorescence detection system on a Hewlett Packard 1100 Series chromatograph (Waldbronn, Germany). The chromatographic separations were carried out on a Luna C18 reverse phase column (150×4.6 mm, 5 µm particle size, Phenomenex, Torrance, CA, USA), connected to a Supelguard LC-ABZ column (20×4.6 mm, 5 µm particle size; Supelco, Sigma-Aldrich). The flow of the mobile phase was 1 ml/min. The wavelength of excitation and emission

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used were 280 and 460 nm, respectively. The ZEA standards were prepared in acetonitrile (purity >99%; Sigma-Aldrich). The limit of detection of the technique was 3 μg/l.

Curve fitting and data processing

Curves representing the amount of bound ZEA as a function of the concentration of ZEA in equilibrium after adsorption were plotted according to mathematical expressions proposed by three theoretical models (Langmuir, Frumkin-Fowler-Guggenheim (FFG) and Hill) that were selected according to the isotherm shapes. Mathematical expressions and parameters of each model are shown in Table 1. Adjustments were made using Origin® version 6.1 software (OriginLab Corp., Northampton, MA, USA).

The quantity of adsorbed ZEA was determined by the following equation:

$$ZEA_{Ads} = \frac{[(ZEA_0 - ZEA_{eq}) \times V]}{m}$$

where 'ZEA_{Ads}' is the amount of adsorbed ZEA per conidia (μg/conidia), 'ZEA_0' is the initial concentration of ZEA in solution (μg/ml), 'ZEA_{eq}' is the concentration of ZEA at equilibrium after adsorption (μg/ml), 'V' is the volume of solution (ml) and 'm' is the number of conidia.

Infrared spectroscopy

For sample preparation, 10 loops of fungal conidia were taken from the colony and placed in a tube. The dead conidia were obtained in the same way for conidial suspensions. The dead conidia were suspended in 1000 μl ZEA (2 μg/ml) solution at pH 2 and pH 6. Samples were homogenised for 30 min and centrifuged at 10,000 rpm for 10 min. The dry pellet was mixed with KCl (1% w/w) and ground in an agate mortar. Finally, the tablet was made under pressure (≈15 ton/cm^2) and applying dynamic vacuum for 30 min.

Measurements for spectra determination were performed in a Nicolet FTIR Impact 400 spectrometer (Thermo Electron Corp., Madison, WI, USA). OPUS software (Bruker Daltonics Inc., Billerica, MA, USA) was used for data acquisition and processing. The spectra were the result of an accumulation of 200 measurements, to increase signal/noise ratio, and were measured between 4,000 and 400 cm^{-1} with a resolution of 4 cm^{-1}.

Transmission electron microscopy

Ten loops of fungal conidia were taken from the colony and placed in a tube. The dead conidia were obtained as described above. The dead conidia were suspended in an Eppendorf vial containing 1000 μl ZEA solution (2 μg/ml) at pH 2 and pH 6. Samples were homogenised for 30 min and centrifuged at 10,000 rpm for 10 min. The dry pellet was processed for transmission electron microscopy (TEM) according to Bozzola and Russel (1999). All samples were pre-fixed in a 0.1 M sodium phosphate buffer (pH 7.2) containing 3% (w/v) glutaraldehyde for 3 h at room temperature, followed by thorough washing with phosphate buffer. Fixed materials were then post-fixed in 1% aqueous osmium tetroxide for 3 h at room temperature. Dehydration of samples was achieved by transferring to vials containing graded water-acetone series (10% steps for the 30-90% range, each of 60 min; 100% for 180 min; and finally 100% overnight). Dehydrated specimens were embedded with EMbed 812 (Electron Microscopy Sciences, Hatfield, PA, USA) and 100% acetone for 24 h, then with EMbed 812 and 1.5% hardening agent DMP-30 at 60 °C for 24 h. Ultra-thin sections (±60 nm) were cut and placed on copper grids, counterstained with saturated uranyl acetate and aqueous lead citrate. The sections were examined

Table 1. Theoretical adsorption models, mathematical equations and adjusting parameters.[1]

Models	Mathematical expression	Parameters
Langmuir	$\beta = \frac{\Gamma}{(\Gamma max - \Gamma)[Tox]}$	Γ_{max}, β
FFG	$\beta = \frac{\Gamma}{(\Gamma_{max} - \Gamma)[Tox]} \exp[-2a\Gamma / \Gamma_{max}]$	Γ_{max}, β, a
Hill	$\frac{1}{KD} = \frac{\Gamma[Tox]^n}{(\Gamma_{max} - \Gamma) + [Tox]^n}$	Γ_{max}, KD, n

[1] FFG = Frumkin-Fowler-Guggenheim; Γ = amount of adsorbed toxin per unit weight of biomass; [Tox] = residual toxin concentration in solution at equilibrium; Γ_{max} = maximum amount of adsorbed toxin per unit weight of biomass; β = adsorption constant; a = parameter measuring the interaction between both adsorbed and in solution toxins; KD = Hill constant; n = Hill cooperativity coefficient of the binding interaction.

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using a transmission electron microscope (Elmiskop 101; Siemens, Erlangen, Germany).

3. Results

Adsorption capacity *in vitro*

A. niger aggregate strain RC084 was chosen on basis of its inability to produce OTA. Figure 2 shows the ZEA adsorption isotherms of dead conidia of strain RC084 at both tested pHs. The isotherms shapes can be classified as S-types; therefore they fit well either with Hill´s equation or with further general treatment according to the FFG equation. The mathematical expressions and the fitting parameters for each adsorption model are shown in Table 1. The theories concerning the Hill and FFG molecular adsorption models are different in nature. Note that K_D tends to β^{-1} when the Hill cooperativity coefficient of the binding interaction (n) tends to one. On the other hand, when the factor 'a' in the FFG equation, which measures the interaction between ZEA at the surface with ZEA molecules in solution, is equal to zero the FFG reduces to the Langmuir equation. The maximum adsorption capacity, measured by Γ_{max}, occurs at pH 6 (2.2×10^{-6} µg/conidia). Setup parameters for the ZEA adsorption in biosorbent for the Hill and FFG models are given in Table 2. As shown by the R^2 values in Table 2, both Hill and FFG models fit the experimental points quite well. The lines displayed in Figure 2 are the ones obtained using the Hill equation.

Infrared spectroscopy

The Fourier-transformed IR spectrometry screened between 4,000 and 400 wavenumbers (cm^{-1}). The spectral windows selected corresponded more or less with the regions known to be characteristic for certain chemical structures: the C-H region dominated by fatty acids (3,050-2,800 cm^{-1}); amide I and amide II, i.e. 1,600-1,700 (carbonyl region, C=O ester absorption) and 1,500-1,600 cm^{-1} (N-H absorption); the spectral region between 1,500 and 1,200 cm^{-1} and polysaccharides (1,200-900 cm^{-1}). The representative IR spectra in the mid-infrared region (4,000-400 cm^{-1}) showing the effect of pH on the ultrastructure of the (dead) conidial walls of *A. niger* are presented in Figure 3.

The IR study was conducted semi-quantitatively to determine the influence of pH on the chemical groups making up the wall of the conidia. Figure 3 shows that the pH did not produce significant changes for the different chemical groups. Only a small shift was observed in the detection of fatty acids according to the wavelength.

Ultrastructure of the conidial wall

Figure 4 shows the ultrastructure of the conidial wall visualised by TEM at both tested pHs. The conidial wall of *A. niger* is composed of several superimposed layers, consisting of a thick electron transparent inner layer and two thin electron dense outer layers; the outermost layer being responsible for the ornamentations of the cell wall. The controls and pH 2 treatment showed the

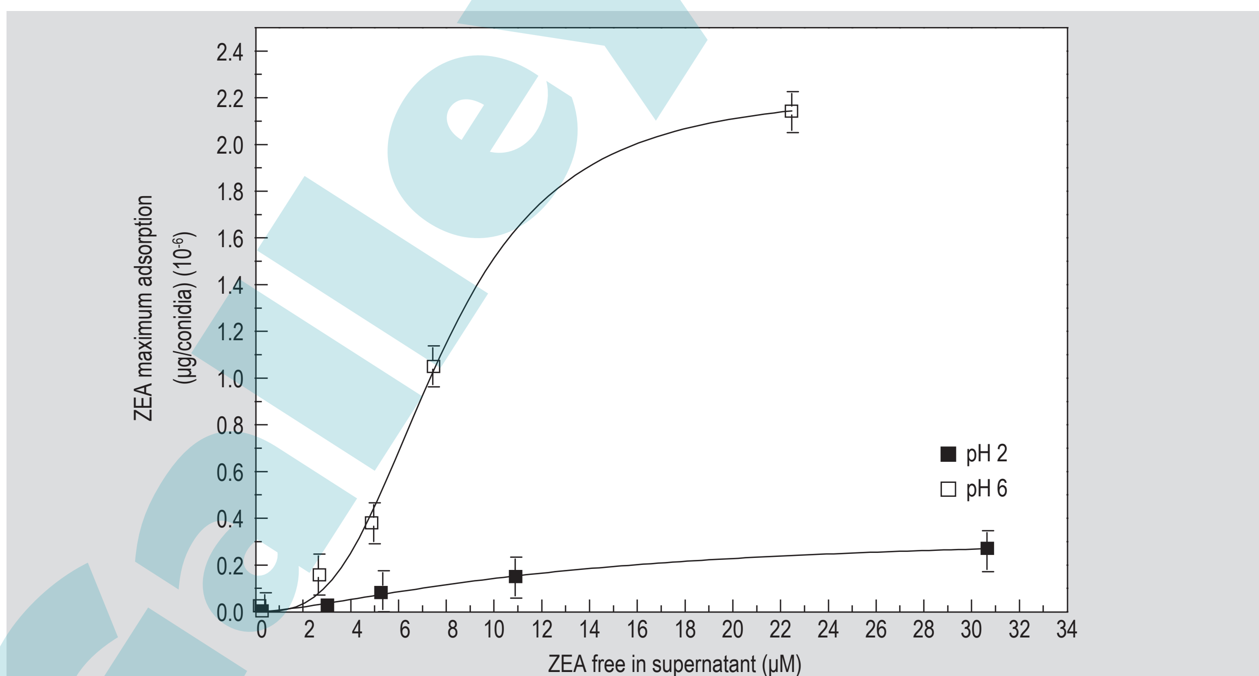

Figure 2. Zearalenone (ZEA) adsorption isotherms by dead conidial walls of *Aspergillus niger* aggregate strain RC084 at pH 2 and 6. The error bars correspond to standard error of the mean.

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Table 2. Setup parameters obtained from zearalenone adsorption isotherms by dead conidia using the Hill and Frumkin-Fowler-Guggenheim (FFG) models.[1]

Model	pH	K_D (M) (10^{-6})	β $(M)^{-1}$ (10^6)	Γ_{max} (µg/conidia) (10^{-6})	n/a	N	R^2
Hill	2	41.6±5.2	0.0240±0.0003	0.34±0.04	1.5±0.3	5	0.996
	6	74.7±0.2	0.0130±0.0001	2.23±0.09	2.1±0.5	5	0.997
FFG	2	-	0.037±0.005	0.34±0.03	0.80±0.30	5	0.998
	6	-	0.022±0.001	2.30±0.02	1.76±0.07	5	0.999

[1] K_D = Hill constant, β = adsorption constant; Γ_{max} = maximum amount of adsorbed zearalenone per conidia; n = Hill cooperativity coefficient of the binding interaction; N = number of points; a = is the FFG parameter measuring the interaction between adsorbed zearalenone molecules with the ones in solution. Each point is the average of two replicates.

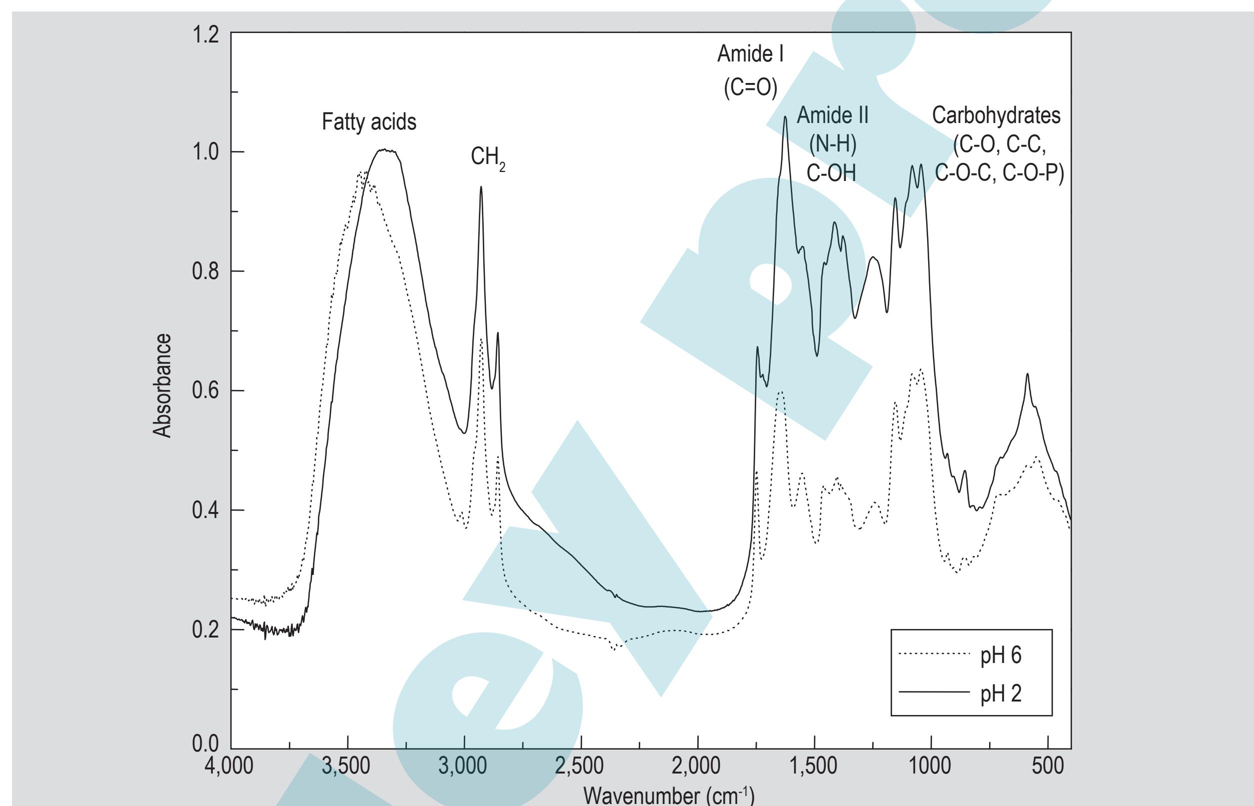

Figure 3. Absorption spectra of dead conidia at pH 2 and pH6 with some characteristic spectral ranges that are dominated by certain chemical structures: fatty acids (3,050-2,800 cm^{-1}), amide I (1,600-1,700 cm^{-1}), amide II (1,500-1,600 cm^{-1}) and carbohydrates (1,200-900 cm^{-1}).

typical echinulate surface of the conidia. However, at pH 6 considerable changes in the organisation of the conidial wall were detected, showing the loss of the outermost electron dense layer.

4. Discussion

The present study assessed the effect of pH on the ultrastructure of the cell wall of dead conidia obtained from non-toxicogenic *A. niger* aggregate strain RC084 and the relationship to ZEA adsorption capacity. Two mathematical models were found to provide the best description of toxin adsorption. The FFG model assumes adsorption on a heterogeneous surface and is applied to either sigmoid or high affinity isotherms. The adsorption model proposed by FFG assumes cooperative, repulsive or non-interacting adsorption mechanisms depending on the adjusting parameter 'a'. It must be considered that β values obtained by the FFG model represent the adsorption constant extrapolated to coating zero. In such a case, all sites are equivalent and no cooperativity is observed in the

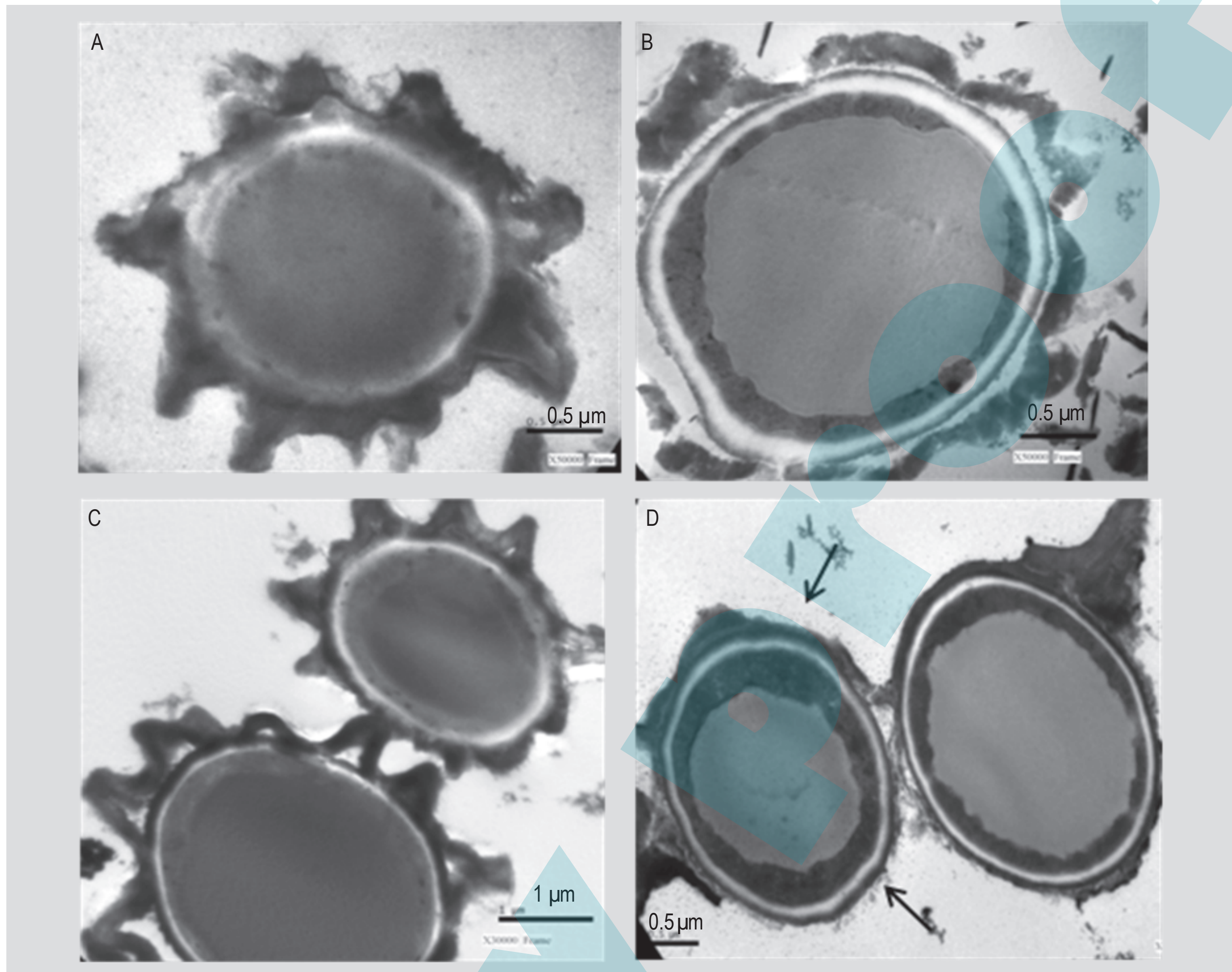

Figure 4. Ultrastructure of *Aspergillus niger* conidia visualised by transmission electron microscopy. (A) control at pH 2; (B) control at pH 6; (c) zearalenone (ZEA) treatment at pH 2; (D) ZEA treatment at pH 6 (on the surface of the conidial wall the lack of the outermost cell wall layer is observed; sometimes it appears free in the surrounding medium).

system. The adsorption constant β differs from the true thermodynamic constant at the adsorption equilibrium, which includes the concentration of displaced solvent from sites at the adsorbent surface (β = Kas $[H_2O]^{-1}$). On the other hand, the Hill model includes the dissociation constant (K_D), the maximum adsorption (Γ_{max}) and the minimum number (n) of binding sites required for cooperative adsorption. Both theoretical models can therefore explain S-type isotherms through cooperate adsorption, however, curve fitting provides either n greater than 1 (Hill equation) or 'a' greater than zero (FFG equation).

Nevertheless, comparable adjustments were obtained with both models; for the purpose of comparison, Hill's model was chosen for further discussion. It has been suggested that this model explains the shape of adsorption isotherms on yeast cell walls and extracts derived from them (Yiannikouris *et al.*, 2003, 2004a). These authors studied ZEA adsorption on *Saccharomyses cerevisiae* cell walls *in vitro* and claim that the Hill equation provided the most accurate parameters. Yiannikouris *et al.* (2004b) also showed that the isolated cell wall of four *S. cerevisiae* strains had some capacity to absorb ZEA and reduce the bioavailability of this mycotoxin in the digestive tract. The adsorption process indicated a cooperative process.

Conidia of black aspergilli have been tested for OTA uptake in grape must and juices (Bejaoui *et al.*, 2005). These authors showed biological removal of OTA by living and heat-treated dead conidia of black aspergilli isolates of *A. niger*, *Aspergillus carbonarius* and *Aspergillus japonicus*, although they did not interpret the results in terms of theoretical models.

The efficiency of ZEA adsorption by conidia was found to be pH-dependent. The effect of pH, such as the change of the conidia typical echinulate surface to a non-echinulate one at pH 6, is difficult to explain. The pH could change

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the composition of the wall of the conidia or the chemical structure of the toxin. The strain adsorption dependence can probably be attributed to differences in chemical composition of the cell wall. However, the IR study only demonstrated changes at the level of fatty acids when they were subjected to different pHs.

Zearalenone is resorcylic acid lactone with an acidic phenol group with a pKa of 10-11 (Moffat *et al.*, 1986). Therefore, no speciation changes could take place at either of the assayed pHs. A neutral resorcylic moiety will render a highly hydrophobic structure. A calculated log P value of 4.94±0.76 for ZEA was previously reported by Navarro Villoslada *et al.* (2007). The higher the log P value, the more hydrophobic the molecule. Considering that conidia are hydrophobic structures, since they have deposits of fat and hydrophobic proteins, such as hydrophobins, presumably present as highly insoluble complexes in the outermost layer of the conidial wall, their interaction with ZEA could be due to the lipophilic nature of this toxin. However, considering the ultrastructural studies by TEM that showed the loss of the outermost electron dense layer of the conidial surface (including the hydrophobins), the hydrophobic interactions themselves do not explain the dependence of adsorption by the conidia on the pH. It is more likely that polar groups in the molecular structure of ZEA form H-bridge interactions with sorbent groups.

In conclusion, this is the first study showing the loss of the outermost electron dense layer responsible for the ornamentations on the conidial surface at pH 6, as determined by IR spectroscopy and TEM. The loss of the outer layer potentiates ZEA adsorption, which can be modelled by the Hill and FFG equations. The use of non-toxigenic strains as adsorbents could be able to prevent the introduction of more mycotoxins to the foodstuffs. Some studies, however, have reported the production of fumonisin B_2 by *A. niger* aggregate strains (Frisvad *et al.*, 2007; Mansson *et al.*, 2010; Mogensen *et al.*, 2010; Susca *et al.*, 2010). Therefore, further studies should also include the analysis of the presence of fumonisin B_2 in dead conidia. Moreover, a detailed analysis of each chemical group involved would elucidate the organisation of the conidial cell wall.

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