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**Conidia of black aspergilli as new biological adsorbents for ochratoxin A in
grape juices and musts**

H. BEJAOUTI^a, F. MATHIEU^a, P. TAILLANDIER^b and A. LEBRIHI^{a*}

Laboratoire de Génie Chimique UMR5503 (CNRS-INPT-UPS)

Département : Bioprocédés et Systèmes Microbiens

^aENSAT/INPT: 1, Av. de l'Agrobiopôle BP32607, Auzeville-Tolosane,

Castanet-Tolosan, 31326, France

^bENSIACET/INPT: 5, rue Paulin Talabot, BP 1301, 31106 Toulouse Cedex 1, France

ABSTRACT

Biological removal of ochratoxin A (OTA) by living and heat-treated dead conidia of black *Aspergillus* isolates representing the species *Aspergillus niger*, *A. carbonarius* and *A. japonicus* in synthetic and natural grape juices was found to be a two-stage phenomenon. Several lines of evidence suggest that the first observed stage was passive, metabolism was not required and OTA adsorption on conidia of black aspergilli could be involved. This removal was fast, without delay just after conidial inoculation both in synthetic and natural grape juices. Moreover, even non-viable, heat-treated conidia were capable of removing OTA. Finally, no OTA degradation products were detected. In the second observed stage, removal of OTA was linked to degradation by live conidia only. Ochratoxin alpha, a degradation product of OTA was detected in the medium after incubation for 30 and 14 hours for biserial (A. *niger* and A. *carbonarius*) and uniserial (A. *japonicus*) black aspergilli, respectively when well-developed mycelium appeared. Comparisons between the three black *Aspergillus* isolates tested showed that A. *carbonarius* detoxified grape juice most effectively. However, this species often produces OTA. A. *niger* and A. *japonicus* isolates were also effective and because those species are not systematically OTA producers, they could be interesting for further OTA detoxification processes in grape juices and musts.

KEYWORDS: ochratoxin A; conidia of black aspergilli; *Aspergillus niger*; *Aspergillus japonicus*; *Aspergillus carbonarius*; adsorption; detoxification; grape juices; musts

* Corresponding author. Telephone: 05 62 19 39 44. Fax: 05 62 19 39 01. E-mail: lebrihi@ensat.fr

INTRODUCTION

Ochratoxin A (OTA) is a naturally occurring secondary metabolite of mould fungi of *Aspergillus* and *Penicillium* genera (1,2). It is affecting agricultural products all over the world and causing harmful effects on human and animal health due to its highly toxic properties (mutagenic, teratogenic, carcinogenic) (3,4). The presence of OTA in grapes and grape products was reported for the first time in 1995 (5). Since then, different studies were undertaken to understand this contamination origin over the world and also in France (6). Several studies (6, 7, 8, 9) showed that *Aspergillus* section Nigri isolates (*A. carbonarius*, *A. niger* and *A. japonicus*) are thought to be the primary source of OTA in grapes, especially *A. carbonarius*. Wines are considered as the second major source of OTA intake after cereals (10) and grape juices are shown to contain more OTA than some wines and so contribute to OTA intake by children (8). Recently, the OTA limit in wine (2 µg/L) is defined by the Commission Regulation (EC) N° 123/2005, of January 2005, amending Regulation (EC) N° 466/2001 as regards ochratoxin A. So, methods for OTA detoxification are highly needed for compliance with tolerances and guidelines to protect consumer health. Different attempts have been made to find measures to combat this mycotoxin. Physical, chemical and biological methods were studied (12), but few of these have practical applications. Some are targeting to degrade OTA in different laboratory media, while some others tried to adsorb it. Different microorganisms belonging to grapes microbiota such as *Aspergillus* section Nigri isolates were reported as able to degrade OTA (13-15), whereas both chemical (fining agents (16)) and biological agents (heat treated yeasts (17)) had the capacity to adsorb it. Although some of those adsorbents could be of interest (17) some others have little effect on the removal of OTA and in many cases the quality of wines is severely damaged (16,18).

For the first time the capacity of conidia of black *Aspergillus* isolates to adsorb ochratoxin A in both synthetic and natural red grape juices was evaluated. Kinetics of OTA

removal and OT α production were done during this survey to clarify the mechanism of OTA removal/degradation by conidia of black aspergilli. Grape juice quality related to colour after conidial inoculation was also assessed.

MATERIALS AND METHODS

Strains. Three *Aspergillus* section Nigri isolates, *Aspergillus carbonarius* SA332 (IMI388497) *A. japonicus* AX35 (IMI389197) and *A. niger* GX312 (IMI388497) isolated on French grapes were used. Identification of different strains of black aspergilli was made using macroscopic and microscopic morphological criteria in accordance with appropriate keys (19-22). They were preserved at the Culture Collection of CABI BIOscience (London, UK). According previous study, *A. carbonarius* SA332 is known to be a weak OTA producer, while *A. niger* GX312 and *A. japonicus* AX35 to be not.

Culturing media. A synthetic grape juice medium (SGM) (ref à donner) and a natural commercial red one were used.

Synthetic grape juice (SGM) was prepared by dissolving: 70 g glucose D(+) (Fisher Labosi (Elancourt cedex, France)), 30g fructose (D-) (Sigma (Saint Quentin Fallavier, France)), 7 g tartaric acid (L-) (Rectapur-Prolabo (Paris, France)), 10 g malic acid (L-) (Fisher Labosi), 0.67 g (NH₄)₂HPO₄ (Prolabo-Rhône Poulenc (Paris, France)), 0.67 g KH₂PO₄ (Acros (Geel, Belgium)), 1.5 g MgSO₄·7H₂O (Acros), 0.15 g NaCl (Fisher Labosi), 0.15 g CaCl₂ (Acros), 0.0015 g CuCl₂ (Prolabo (Paris, France)), 0.021 g FeSO₄·7H₂O (Riedel-de Haën (Seelze, Germany)), 0.0075 g ZnSO₄·7H₂O (Fisher Labosi) and 0.05 g hydrated catechin (Sigma); in 1 L distilled water and the pH was adjusted at 4 with KOH 2N.

SGM was supplemented with OTA at 2 mg/L, while grape juice was contaminated at two concentrations of 2 mg/L and at 10 µg/L.

Preparation of spore suspensions. Living spores of the three *Aspergillus* section Nigri isolates were obtained from mycelium grown on CZAPEK Yeast extract Agar (CYA)

medium, at 25°C, aged 7 days. Before use, spores were carefully washed with physiological water to remove any contaminants. Dead conidia were obtained through boiling living spores in distilled water for 15 minutes.

Adsorption and degradation conditions. Kinetic studies of OTA adsorption, OTA degradation and OTA production were performed for the three *Aspergillus* section Nigri isolates, *A. carbonarius* SA332, *A. japonicus* AX35 and *A. niger* GX312, in 20 mL of SGM containing 2mg/L of OTA, at 25°C, under agitation (240 rpm). As control to calculate OTA removal percentage, we used SGM containing 2mg/L of OTA and inoculated with a water blank without conidia. So this dilution was not significant. All assays were performed in triplicates.

Effect of initial conidial concentration on OTA removal was conducted with *A. japonicus* in SGM containing 2mg/L of OTA. Four different conidial concentrations, 10^4 , 10^6 , 10^7 , and 10^8 conidia/mL were chosen.

The OTA removal capacity of the three *Aspergillus* section Nigri isolates was undertaken in 20 mL of synthetic and natural red grape juices. Each medium was inoculated with conidial suspensions of each isolate to give a concentration of 10^7 conidia/mL.

For all samples, after removal of conidia or fungal mycelium, 1 mL from each supernatant was filtered (0.22 µm) and without previous clean-up step analysed by HPLC.

Conidial germination. During adsorption studies in SGM inoculated with the three *Aspergillus* section Nigri isolates, conidial germination was followed (NIKON Eclipse Microscope E600) and photos were taken at 7, 11, 14, 20 and 24 h of incubation time to determine the physiological state of spores. Photos were analysed by the image program VISOLOG 5 (NOESIS, Quebec, Canada) to follow conidial germination.

Colour of red grape juice. To assess the tint of the red grape juice before and 5 minutes after introducing fungal conidia, the optical density (OD) was measured at 420 nm

(OD₄₂₀) and at 520 nm (OD₅₂₀). The tint (T) was determined by the ratio of OD₄₂₀ to OD₅₂₀ ($T = OD_{420}/OD_{520}$) (23).

Detection and quantification of OTA and OT α OTA and OT α were detected and quantified by reversed-phase high performance liquid chromatography. The HPLC apparatus consisted of a solvent delivery system and fluorescence ($\lambda_{ex} = 332$ nm; $\lambda_{em} = 466$ nm) and UV detectors. The analytical column used was a 150 x 4.6 mm Uptisphere 5 μ C18 ODB fitted with a guard column of 10 x 4 mm. The mobile phase consisted of a mixture of HPLC grade acetonitrile/water/acetic acid (100/99.8/0.2) at a flow rate of 1 mL/min and the column temperature was at 30°C. Kroma 3000 (Biotek) was the data acquisition system. Injections were made with an autoinjector (BIO-TEK, Milan, Italy) and the injection volume was of 80 μ L. Ochratoxin A was identified by its retention time (33 min) according to a standard obtained from Sigma (Steinheim, Germany) and quantified by measuring the peak area. The detection limit was 0.025 μ g/L.

The OTA removal percentages were calculated according to the following equation: $100 \times [1 - (\text{peak area of OTA} / \text{peak area of OTA in control})]$. OT α was identified at 17 min according to a standard prepared by total degradation of OTA by carboxypeptidase A (EC3.4.17.1) from bovine pancreas (Sigma, type II-PMSF). OTA and OT α were quantified by measuring the peak area and using standard solutions.

Statistical analysis. All analysis were done in triplicate. SPSS (Headquarters (Chicago, Illinois, USA)), version 11.5.1, for windows was used for the statistical analysis of the data. Significant differences in the mean values were reported at P values of <0.05 .

RESULTS

Ochratoxin A removal in synthetic grape juice (SGM). A synthetic grape juice, initially contaminated with OTA at 2 mg/L, was inoculated with three *Aspergillus* section

Nigri isolates, *Aspergillus niger*, *A. japonicus* and *A. carbonarius* at a concentration of 10^7 conidia/mL. It was incubated at 25 °C under agitation and samples from SGM were taken over 57 hours. **Figure 1** represents OTA removal and OTα production versus time for each species tested. Just after conidia of black aspergilli introduction in SGM and well mixing, decreases in OTA amounts were instantly observed for the three *Aspergillus* section Nigri isolates. Those decreases were about 10% for *A. niger*, 28% for *A. japonicus* and 45% for *A. carbonarius*. Until 30 hours for *A. niger* and *A. carbonarius* and 14 hours for *A. japonicus*, no OTA degradation products were observed. OTα was only detected beyond those times. For two *Aspergillus* section Nigri isolates, *A. japonicus* and *A. carbonarius*, slight increases in OTA amounts were also sometimes observed could indicate a partial release of this mycotoxin in the medium.

Among the three *Aspergillus* section Nigri isolates tested in this study, *A. japonicus* was selected to test the effect of initial conidial concentration (10^4 , 10^6 , 10^7 and 10^8 conidia/mL) on OTA removal from SGM (**Figure 2**). We noted that the higher concentration of conidia introduced in the medium was, the greater the OTA removal. Thus, with 10^4 conidia/mL, OTA adsorption was of 15% to reach 75% with 10^8 conidia/mL.

Conidial germination. OTA adsorption surveys in SGM permit us to observe the physiological states of conidia of *A. carbonarius*, *A. niger* and *A. japonicus* (photos not shown). At 14 hours, conidia of *A. carbonarius* and *A. niger* could either be in a dormant state, swollen or have a germ tube. For conidia of *A. japonicus*, swelling and formation of germ tubes earlier began at around 11 hours. So beyond 11 hours for *A. japonicus* and 14 hours for *A. niger* and *A. carbonarius*, long hyphae were developed and aggregated forming dense fungal mycelium.

Ochratoxin A removal in a natural grape juice. The red natural grape juice used in this assay was contaminated at two different concentrations of OTA: 2 mg/L and 10 µg/L.

176 Living and dead spores from three *Aspergillus* section Nigri isolates were inoculated in the
177 grape juice at the concentration of 10^7 conidia/mL. Samples from grape juice were taken over
178 two hours and analysed for OTA content.

179 According to the initial OTA concentration in the grape juice (2 mg/L or 10 µg/L) and to
180 black *Aspergillus* isolates tested, differences between OTA adsorption patterns were noted
181 (**Figure 3 and Figure 4**). As soon as conidia of black aspergilli were added to the grape juice
182 contaminated at 2 mg/L (**Figure 3**), OTA was immediately adsorbed on living conidia. So, in
183 a few second later, removal of OTA was already of around 30% by conidia of both *A.*
184 *japonicus*, and *A. niger* and reached 55% by conidia of *A. carbonarius*. After this
185 instantaneously adsorption, amounts of OTA were kept constant until 120 minutes. For heat-
186 treated conidia the same removal pattern was observed, but OTA removal was improved and
187 was about 41.5 % for *A. japonicus*, 47.5 % for *A. niger* and 66.5 % for *A. carbonarius*.

188 For the grape juice contaminated with OTA at 10µg/L (**Figure 4**), OTA removal by living or
189 heat-treated conidia of *A. japonicus* and *A. carbonarius* was instantly observed and reached
190 around 80%. Then, OTA removal was kept constant till 120 minutes, except with heat-treated
191 conidia of *A. carbonarius* on which OTA adsorption reached 96% after 75 minutes. A
192 particular behaviour was observed with both living and heat-treated conidia of *A. niger*,
193 especially in the first minutes. No immediately adsorption occurred on living conidia and only
194 15% on dead conidia. However, after 40 minutes OTA adsorption on living conidia was
195 around 60% to reach 70% after 60 minutes. With heat-treated conidia, OTA adsorption was
196 improved to 90% after 120 minutes.

197 We also determined the impact of conidia of black *Aspergillus* isolates on grape juice colour.
198 The tint of the grape juice was assessed before and after introducing living fungal conidia.
199 The tint of the control was about 1.18, whereas it was 1.46, 1.47 and 2.19 after addition of

conidia of *A. japonicus*, *A. niger* and *A. carbonarius* respectively, indicating a darker colour of the red juice after addition of black conidia.

DISCUSSION

OTA adsorption studies on conidia of black aspergilli, in both synthetic and natural red grape juices contaminated at 2 mg/L and 10µg/L, showed that this is an instantaneous removal of OTA in many cases for the three *Aspergillus* section Nigri isolates, except for *A. niger* introduced in grape juice contaminated at 10 µg/L. No degradation product like OTα was detected during stages of conidial dormancy, swelling and germ tube but occurred over 30 hours for *A. niger* and *A. carbonarius* and 14 hours for *A. japonicus*. Heat-treated conidia were also able to remove OTA from the natural red grape juice. Those observations suggest that OTA removal is well an adsorption phenomenon. The OTA adsorption was little higher in natural red grape juice than in SGM. This could be related to the different composition of the two media. SGM is closed to grape juice at early veraison, hence would contain less sugar and more acid. Two different concentrations were tested, 10µg/L and 2mg/L. The first one is very closed to a concentration naturally present in some musts that needed biological detoxification. The second, 2 mg/L, obvious it was 1000-times greater than the 2 µg/L limit in wine, allowed, *in vitro*, to better understand the mechanism of OTA removal/degradation (i.e. easier detection of eventual degradation products) and to determine importance of OTA/conidia ratio. Results showed that the efficacy of OTA adsorption was better with a small OTA/conidia ratio even in the case of the biggest conidia of *A. carbonarius*. OTA adsorption seems to be related both to the size and to the amounts of conidia. For *A. carbonarius* conidia, the biggest one, 10⁷ conidia/mL adsorbed 50% of OTA versus only 30% with 10⁷ conidia/mL of *A. japonicus*, the smallest. To increase the efficacy of *A. japonicus* conidia, it is necessary to increase the concentration to 10⁸ conidia /mL.

224 To understand interactions involved in this adsorption, properties of both OTA and conidia
225 surfaces should be described. According to the literature, mycotoxin binding was reported as
226 function of their structural characteristics (24), their molecular size and their physico-
227 chemical properties (16). OTA is a complex organic compound, consisting of chlorine
228 containing dehydroisocoumarin linked through the 7-carboxyl group to 1- β -phenylalanine
229 (25). Phenol and carboxyl are the main functional groups of this molecule (26) that could be
230 involved in different adsorption mechanisms. First, OTA is considered with zearalenone as
231 the less polar mycotoxins and could then be bound on hydrophobic surfaces (27) through the
232 phenol group and via interactions of two- π -electron orbital (28). This was already shown for
233 OTA adsorbents like hydrophobic transformed zeolites (29). Second, OTA acidic function is a
234 weak acid with a pKa value for the carboxyl group of the phenylalanine moiety of 4.4 (30).
235 OTA is then partially dissociated at pH 4.0 of grape juices and carries a positive charge
236 introduced by the amine function (NH_3^+).

237 For conidia, and particularly the airborne types, a hydrophobic nature was reported (31). This
238 hydrophobicity could be due to proteins called hydrophobins (32,33) or to lipids previously
239 found on *Botrytis fabae* conidia (31). Negatively charged carbohydrates were also found on
240 the surface of *Aspergillus fumigatus* conidia (34) Those properties has intervened in adhesion
241 phenomena of living (35) or dead (36) conidia on different natural surfaces (roots (35), fruit
242 cuticles (36))

243 According to all above properties reported in literature, adsorption phenomenon of OTA on
244 conidia of black aspergilli could be related to hydrophobic interactions. The global positive
245 charge of OTA molecule in acid media (grape juices) could also interact with negatively
246 charged molecules found on fungal conidia. This interaction is likely to be non specific, as
247 many conidia are able to bind non-specifically or without requiring chemical compounds.

According to some of our results, increases in OTA amounts were observed during the adsorption phenomenon. This could be a partial release of the initially bound mycotoxin. This release was also observed for *Fusarium oxysporum* conidia initially adsorbed on root surfaces (35) and during the adsorption of different metal toxicants on fungal conidia (37). Perhaps conidial interactions were easily broken, as conidia adhesion was previously reported as a relatively weak attachment for *Botrytis cinerea* (36).

After OTA adsorption by conidia of black aspergilli, OTA degradation took place by well-developed mycelium after 14 hours for *A. japonicus* and 30 hours for both *A. niger* or *A. carbonarius*. Thus, OT α , an OTA degradation product, also observed in different previous studies (13,15), increased while OTA disappeared from the grape juice. So, two stages were involved in OTA detoxification by *Aspergillus* section Nigri isolates : adsorption then degradation. Commercially, the only stage very interesting is adsorption because it is fast, avoiding long time contact between fungi and grape juice. So potentially commercial OTA detoxification by using conidia of black aspergilli must be completed before the mycelium is well-developed and degradation starts. Indeed, production of degradation product like OT α even reported less toxic (38), still be harmful for consumer health. Moreover, degradation is useless for detoxification of grape juice in so far as by this stage, the fungus is actually using up all the grape sugars for its own growth. We also noted that even the difference between dead and live conidia was not so important, dead conidia consistently bound more OTA at 2mg/L. In view of commercially detoxification, dead conidia could also only be used to completely prevent germ tub formation and degradation phenomenon.

Among three *Aspergillus* section Nigri isolates tested, conidia of *A. carbonarius* were in many cases the most efficient in adsorbing OTA, regardless of the media used and the OTA concentration tested. In fact, conidia of *A. carbonarius* had the largest diameter (7-10 μ m) compared to conidia of *A. niger* (3.5-4 μ m) (39) and those of *A. japonicus* (5 μ m) (19) and so

presented higher surface to bind OTA. However, *A. carbonarius* could not be so interesting for practical detoxification, as it hardly severely deteriorated grape juice colour and because it is known to be particularly significant in the production of OTA on French grapes (6,40) but elsewhere (41-43). We noted that *A. japonicus* and *A. niger* conidia less deteriorated the grape juice colour than the *A. carbonarius* conidia. For *A. niger* and *A. japonicus*, OTA adsorption patterns were different according to the media used and to OTA concentrations tested. However, those two species could be interesting for OTA removal in grape juices and musts as the first has the GRAS label, and the second, frequently used in fungal biotechnology, has been reported as ochratoxigenic only a few times (7,44,45).and both of them less deteriorated grape juice colour than *A. carbonarius*.

Finally the risk of using conidia of OTA producing species in OTA detoxification process (i.e having both OTA production and degradation products like OT α in the medium to be detoxified) could be drastically eliminated by using OTA non-producing species and dead conidia allowing dormancy state to be prolonged.

For a routinely use of conidia of black aspergilli for lowering OTA levels in grape juices, two points remains to be improved. The first is to do a treatment of spore to eliminate their black colour which affect final juice colour and second, to think about an immobilization of the spores to facilitate their use in industrial scale. OTA decontamination of wine by this process could be promising as so far as the efficiency of conidia of black aspergilli is demonstrated.

SAFETY

Ochratoxin A is a toxic compound that needs to be manipulated with care and with appropriate safety precautions. Decontamination procedures for laboratory wastes have been reported by the International Agency for Research on Cancer (IARC) (46).

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416

FIGURE CAPTIONS

Figure 1. Ochratoxin A removal and ochratoxin α appearance during incubation of the three *Aspergillus* section Nigri isolates, *A. niger* GX312, *A. carbonarius* SA332, *A. japonicus* AX35 in SGM medium contaminated with OTA at 2 mg/L at a concentration of 10^7 conidia/mL. The amounts of OT α are relative to initial OTA concentrations.

Figure 2. OTA removal by *A. japonicus* AX35 in SGM (contaminated with OTA at 2mg/L) inoculated at different concentrations of live conidia (10^4 , 10^6 , 10^7 , and 10^8 conidia/mL).

Figure 3. OTA removal by living and dead conidia of the black aspergilli tested at 10^7 conidia/mL (*A. niger*, *A. japonicus* and *A. carbonarius*) in a natural red grape juice contaminated with OTA at 2 mg/L.

Figure 4. OTA removal by living and dead conidia of the black aspergilli tested at 10^7 conidia/mL (*A. niger*, *A. japonicus* and *A. carbonarius*) in a natural red grape juice contaminated with OTA at 10 μ g/L.

FIGURES

Figure 1

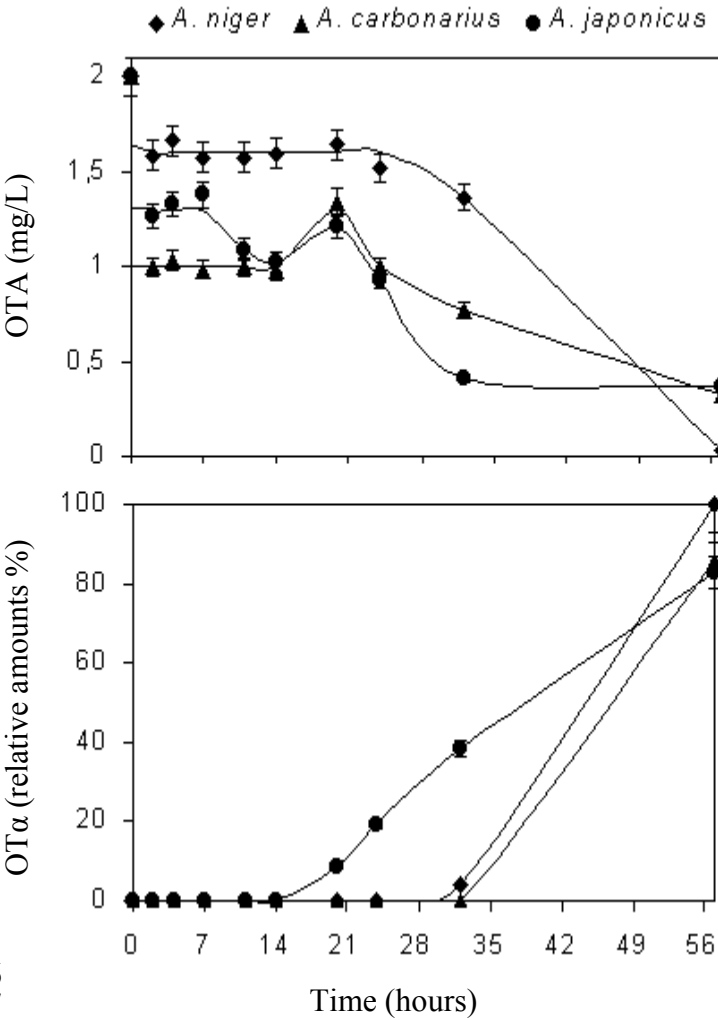


Figure 2

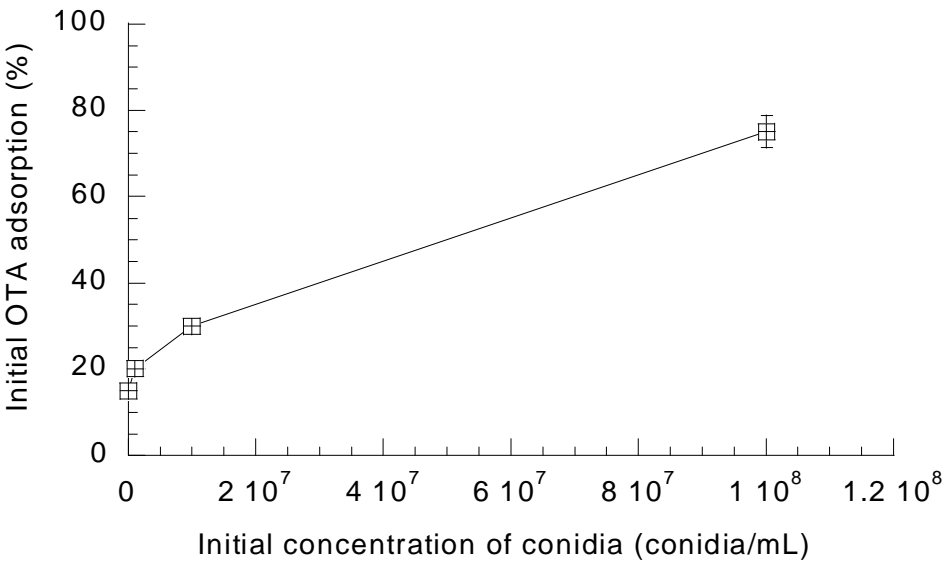


Figure 3

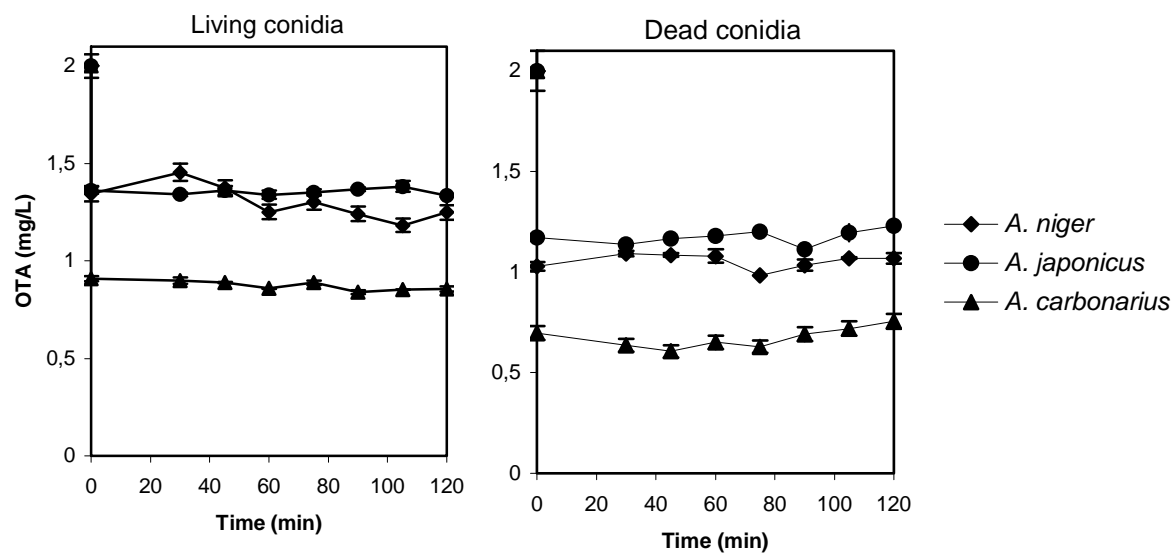


Figure 4

