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IV International
Specialized Conference
on Sustainable
Viticulture: Winery
Wastes and Ecologic
Impact Management
(Winery2006)





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Assessing the Degradation of Ochratoxin A Using a Bioassay: The Case of Contaminated Winery Wastewater

R. Nogueira*, I. Estevinho*, L. Abrunhosa*, C. Mendonça*, P. Machado*, M. Carballa**, A. G. Brito* and A. Venâncio*

* Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal (E-mail: regina@deb.uminho.pt; agbrito@deb.uminho.pt; avenan@deb.uminho.pt)

** School of Biochemical Engineering, Pontificia Universidad Católica de Valparaíso. General Cruz 34, Valparaíso, Chile (E-mail: marta.carballa@ucv.cl).

Abstract In vineyards the presence of certain fungi may lead to the production of the mycotoxin ochratoxin A (OTA) and subsequent contamination of grapes and wine. Furthermore, winery wastewaters contaminated with OTA may represent an environmental hazard. Therefore it is imperative to assess the fate of this mycotoxin in conventional wastewater treatment systems.

The aim of the present work in this context is to assess the biological degradation of OTA. Experimental work was carried out in batch experiments with initial OTA to biomass concentration ratios (S0/X0) of 1.4 $\mu\text{g mg}^{-1}$, 7.4 $\mu\text{g mg}^{-1}$ and 11.9 $\mu\text{g mg}^{-1}$. The assays were inoculated with activated sludge biomass unadapted to the substance under examination. The proposed bioassay demonstrates that OTA concentrations up to 100 $\mu\text{g L}^{-1}$ can be degraded by microbial activity in activated sludge.

Keywords Activated sludge; bioassay; biodegradability; ochratoxin A; OECD; winery wastewater

Introduction

Ochratoxin A (OTA) is a mycotoxin, that is a secondary metabolite produced by certain filamentous fungi. Chemically speaking, OTA is a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin, linked through its 7-carboxy group to L- β -phenylalanine by an amide bond (van der Merwe, 1965, Figure 1). Only two fungal genera have species able to produce OTA: *Aspergillus* and *Penicillium*. It is considered one of the main mycotoxins hazardous for human health, with nephrotoxic, nephrocarcinogenic, teratogenic, and immunosuppressive properties (CAST 2003). The presence of OTA producing fungi in vineyards may lead to contamination of grapes before the harvest and consequently to contamination of the wine produced and the wastewater generated in the production process. Wineries generate large volumes of wastewater mainly from various washing operations during the crushing and pressing of grapes as well as from rinsing of fermentation tanks, barrels and other equipment (Brito *et al.*, 2006).

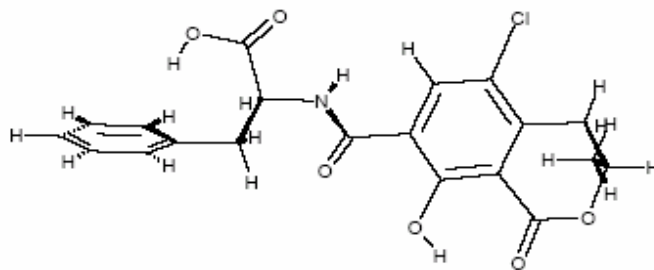


Figure 1: Chemical formula of OTA.

The use of batch cultivation in biological wastewater treatment research has been described in literature. Chudoba *et al.* (1992) have shown that the most important parameter in batch cultivation of mixed cultures is the ratio of the initial substrate concentration (S_0 , measured as chemical oxygen demand) to the initial biomass concentration (X_0 , measured as mixed liquid suspended solids). The ratio between S_0 and X_0 determines whether or not cell multiplication will take place during the exogenous substrate removal stage. When it is low (below 2-4, depending on the mixed culture's history) no cell multiplication occurs during exogenous substrate removal, and biomass increase is mostly due to the synthesis of storage polymers. Under high S_0/X_0 conditions, however, more energy is spent for cell multiplication, which results in a higher amount of substrate being oxidized.

Little is known about mycotoxin biodegradation, in particular the fate of these metabolites in wastewater treatment systems and the toxic effects of contaminated wastewater discharges into natural waters. The strategy for testing the biodegradability of chemicals adopted by the Organization for Economic Co-operation and Development (OECD) consists of three levels (Painter, 1995): ready biodegradability tests or screening tests, inherent biodegradability tests and, simulation tests. Depending on the test results, three levels of degradability are distinguished: readily biodegradable, inherently biodegradable, and persistent. The tests are batch or (semi-) continuous and they differ in the amount of biomass and substrate concentration used.

The test for the “ready biodegradability” level is relatively stringent as the incubation time is limited. Furthermore, the inoculum is of low density and unadapted to the chemical being tested, since it is taken directly from environmental sources such as activated sludge, sewage effluents, surface waters and soils or from a mixture of these (Blok and Balk, 1994; OECD 1992). The test itself consists in adding the test chemical as the only carbon source to an inoculated mineral medium. After initial biodegradation is established, a so called lag phase is allowed to pass, in which 10% of the chemical is consumed. The chemical can be classified as “readily biodegradable” if after the end of the lag phase the subsequent extent of degradation exceeds a defined ‘pass’ level within 10 d (10-day window). The required value for the pass level depends on the method used for analyzing the amount of residual chemical: 80% for specific analysis, 70% for DOC¹ and 60% for ThOD² or ThCO₂³ analyses (Painter, 1995). If a chemical does not pass the “ready”-level test, either degradation starts too late or it occurs too slow. The different OECD tests for ready biodegradability are: DOC die-away, CO₂ evolution, Manometric respirometry, Modified OECD Screening, Closed bottle and MITI(I) (OECD 1992). To test for “inherent” biodegradability, a higher concentration of inoculum and a longer incubation time are used than in the “ready” tests. The Zahn-Wellens test lasts for 28 d (Painter, 1995) while the semi-continuous activated sludge test (SCAS) has a maximal duration of 12 to 26 weeks (OECD, 1984). To assess the biodegradability potential that is relevant in the environment, deviations from environmental conditions should be avoided in the lab tests. When a chemical has not passed a “ready”-level test, but passes a test for inherent biodegradability, an additional simulation test may be started. The OECD simulation test is based on the OECD confirmatory test for surfactant biodegradation and uses the Husmann apparatus (OECD, 2001). This test basically simulates a wastewater treatment plant process

¹ Dissolved Organic Carbon

² Theoretical Oxygen Demand

³ Theoretical CO₂ production

with representative hydraulic and sludge retention times.

The purpose of this study was to evaluate a bioassay according to OECD standards in order to classify the degradation potential of ochratoxin A from winery wastewater.

Materials and Methods

OTA degradation bioassay

In order to assess the biological degradation of ochratoxin A (OTA), a number of batch assays was conducted. In one line of experiments synthetic substrate solutions were utilized, which were contaminated with OTA concentrations ranging from 5 $\mu\text{g L}^{-1}$ to 100 $\mu\text{g L}^{-1}$. Acetate was added to represent soluble organic matter. The synthetic solution was composed as follows: 643 mg L^{-1} $\text{NaCH}_3\text{COO}\cdot 3\text{H}_2\text{O}$, 130 mg L^{-1} NH_4Cl , 210 mg L^{-1} NaHCO_3 , 44 mg L^{-1} KH_2PO_4 and trace element solution 1 mL L^{-1} . The pH was adjusted to 7.5 (Vishnic and Santer, 1975). A second line of experiments was conducted with real winery wastewater contaminated with defined amounts of OTA.

Experiments were performed in erlenmeyer flasks with an effective volume of 10 mL, placed in an orbital shaker at 20 °C. Two control assays were included in the procedure: uncontaminated assays (without OTA) and assays with OTA as a single carbon source. The assays were always conducted in duplicate. Activated sludge from a winery wastewater treatment plant was used as inoculum, the medium initial biomass concentration being 10 mg L^{-1} determined as volatile suspended solids (VSS). Degradation progress of ochratoxin A and acetate was assessed in samples filtered with a 0.22 μm filter. To evaluate the effect of biosorption on OTA removal, batch experiments were also conducted with sludge previously inactivated by autoclaving at 121°C. Abiotic losses like adsorption to glass were also checked. OTA was purchased from Sigma, in crystalline form. A stock solution of 1 mg L^{-1} was prepared in toluene–acetic acid (99:1) and stored at -20 °C. The OTA concentration of this solution was determined accurately by UV spectrophotometry at 331 nm.

Analytical methods

Ochratoxin A determination was conducted by high-performance liquid chromatograph on samples pre-filtered with glass fiber filters. Ochratoxin A quantification was done according to Abrunhosa *et al.* (2002). The samples were injected directly into a reverse phase HPLC equipped with a Jasco FP-920 fluorescence detector (330 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separations were performed on a C18 column (Waters Spherisorb ODS2, 4.6×250 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 mL/min and consisted of an isocratic program as follows: acetonitrile/water/acetic acid (99:99:2, v/v). The injection volume was 100 μL .

Acetate was analyzed by using a HPLC system (KNAUER) with a 210 nm-UV-detection and an organic acid column (PL Hi-Plex H, 300 mm x 7.7 mm, Polymer Laboratories) at 65 °C. The mobile phase consisted of an aqueous H_2SO_4 solution (2 mmol L^{-1}) at a flow rate of 0.7 mL min^{-1} .

Activated sludge volatile suspended solids were determined according to Standard Methods (APHA, 1989).

Results and Discussion

In order to assess biosorption and adsorption effects on the experimental results, blank assays were conducted with identical equipment as the subsequent experiments, but with an inoculum inactivated by autoclaving. As illustrated by Figure 2, no diminution of the initial OTA concentration occurred during 90 h, so that both biosorption and adsorption effects could be excluded.

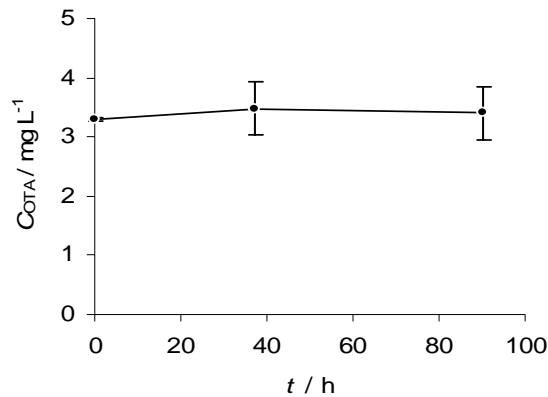


Figure 2: OTA-concentration over time without biodegradation (inoculum inactivated by autoclaving).

Figure 3 represents actual biodegradation assays both with synthetic substrate solution and a real winery effluent. The diagram illustrates that the OTA degradation rates (corresponding to the curves' slopes) increased with the initial contaminant concentration, both for synthetic and real wastewater. In Figure 3a, the curves' sigmoidal shapes indicate that in accordance with the findings of Chudoba *et al.* (1992) the OTA degradation was accompanied by cellular multiplication.

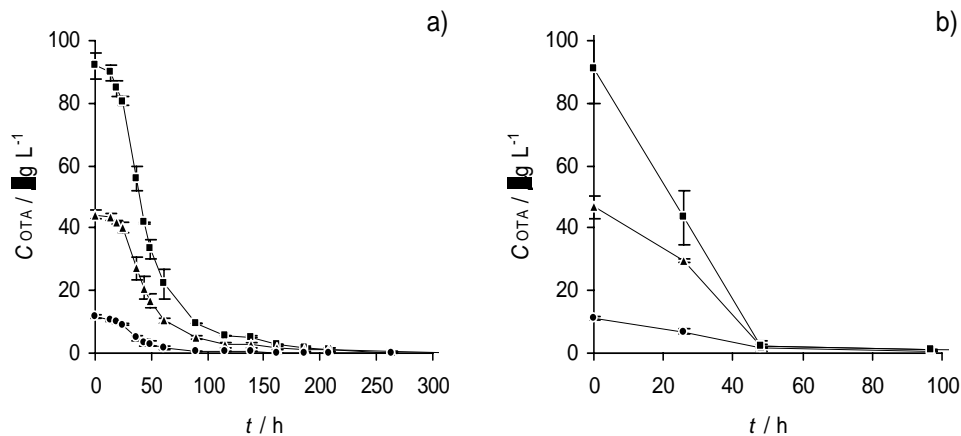


Figure 3: Ochratoxin A biodegradation in different samples: a) synthetic substrate solution; b) winery effluent. Error bars present standard deviations. The curves were obtained at S₀/X₀ ratios of (●) 1.39 μg mg⁻¹, (▲) 7.44 μg mg⁻¹, and (■) 11.9 μg mg⁻¹.

Figure 4 depicts the specific degradation rate of OTA as a function of the S0/X0 rate. The specific degradation rate was calculated as the ratio between maximum removal speed and the initial biomass concentration. According to the represented results the specific degradation rate is directly proportional to the S0/X0 ratio.

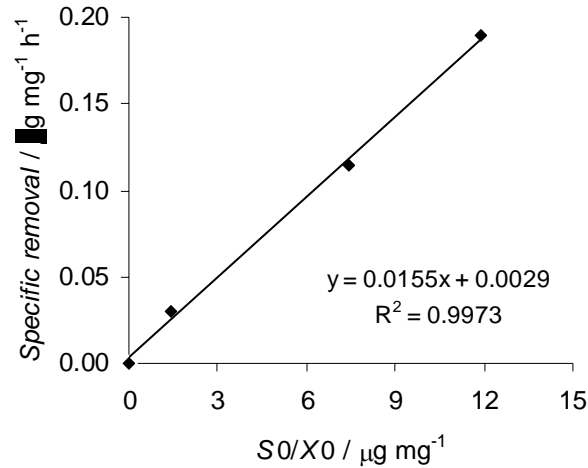


Figure 4. Specific OTA removal as a function of S0/X0, obtained with synthetic substrate solution.

As illustrated by Figure 5, the experiments described above also demonstrated that OTA concentrations up to $100 \mu\text{g L}^{-1}$ had no significant effect on the biological removal of soluble organic matter (here represented by acetate).

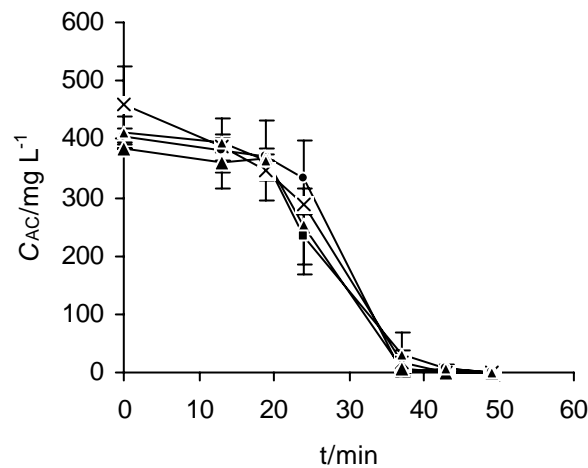


Figure 5. The effect of OTA on degradation of soluble organic matter (acetate): the essays were carried out at increasing initial OTA concentrations, respectively, (×) $0 \mu\text{g L}^{-1}$, (●) $12 \mu\text{g L}^{-1}$, (▲) $44 \mu\text{g L}^{-1}$, (■) $92 \mu\text{g L}^{-1}$. Error bars present standard deviations.

As stated earlier, the evaluation of the biodegradability level of ochratoxin A with respect to OECD standards was one objective of the present study. Since a specific analysis of OTA was utilized, the OECD classification as "readily biodegradable" calls for a "pass-level" of 80% to be removed in the bioassay. This removal is required to occur in a specific bioassay with OTA as the sole carbon source, and within 10 d after the initial lag phase. Figure 6 represents the corresponding conditions. As illustrated, a 80 % removal occurs within less than 240 h (10 d) for both initial concentrations, so that OTA may indeed be considered “readily biodegradable” according to the OECD standard.

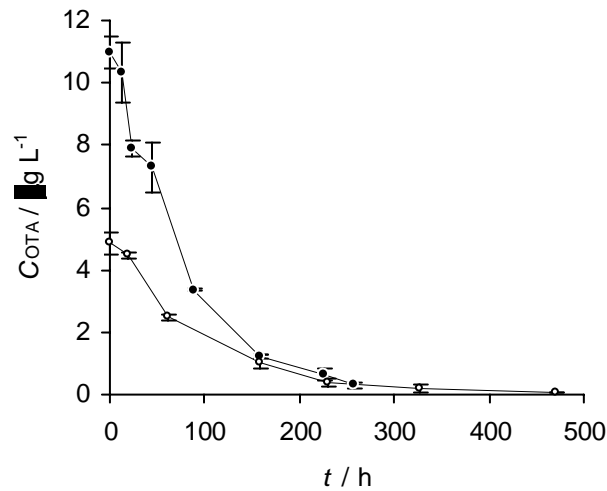


Figure 6: Ochratoxin A degradation as sole carbon source. The assays were conducted with synthetic substrate solution without acetate. Error bars present standard deviations.

As ochratoxin A degradation occurred both with and without the presence of soluble organic matter (acetate), it was of evident interest to assess the influence of this acetate on the respective degradation speed. A comparison of two respective degradation curves (Figure 7) illustrates that in the presence of acetate the OTA removal occurs significantly faster. This effect is probably due to the higher accumulation of biologically active biomass brought forward by the presence of acetate as a readily degradable carbon source.

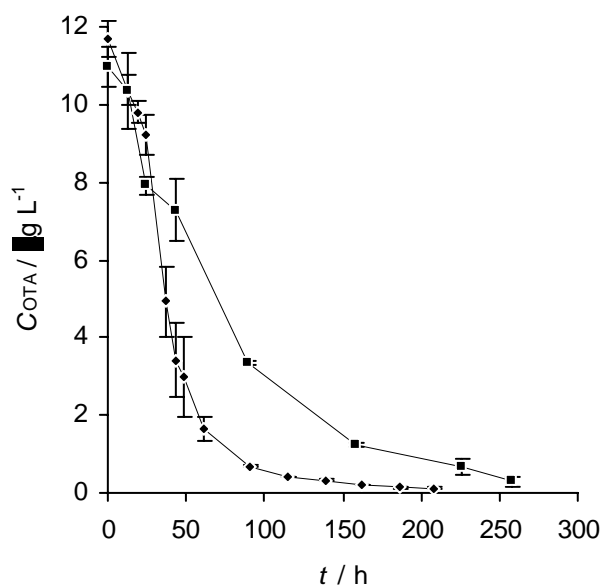


Figure 7: Illustration of acetate’s effect on ochratoxin A degradation. Assays were conducted with synthetic substrate solution with acetate (●) and without acetate (■). Error bars present standard deviations. The curves were obtained at S0/X0 ratios of (■) $1.11 \mu\text{g mg}^{-1}$ and (●) $1.38 \mu\text{g mg}^{-1}$.

The ready biodegradability of ochratoxin A, as demonstrated in this study, can be regarded as a relieving result. It would be desirable, however, in further research to detect its possible biodegradation sub-products, such as ochratoxine α , and to determine their toxicological relevance and subsequent fate.

CONCLUSIONS

The following main conclusions can be drawn from the present study:

- Ochratoxin A concentrations of up to $100 \mu\text{g L}^{-1}$ were degraded by activated sludge microorganisms;
- The presence of OTA in this concentration range had no significant effect on the biological removal of organic carbon (acetate);
- Microorganisms present in the activated sludge were able to degrade ochratoxin A even as single carbon source within OECD standard conditions, OTA thus may be considered as “readily biodegradable”;
- The proposed procedure could be used as a standard bioassay to assess the removal of other mycotoxins from winery effluents.

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References

- Abrunhosa L., Serra R. and Venâncio A. (2002). Biodegradation of Ochratoxin A by Fungi Isolated from Grapes. *Journal of Agriculture and Food Chemistry*, **50** (25), 7493–7496.
- Standard methods for the examination of water and wastewater*. (1989). 16th edn, American Public Health Association/American Water Works Association/Water Environment Federation, New York, USA.
- Blok J. and Balk F. (1994). Commission of the European Communities: guidance document for the interpretation of biodegradability test data. Technical report, BKH Consulting Engineers, Delft, The Netherlands.
- Brito A. G., Peixoto J., Oliveira J. M., Oliveira J. O., Costa C., Nogueira R. and Rodrigues A. (in press). Brewery and winery wastewater treatment: some focal points of design and operation”, In: *Utilization of By-Products and Treatment of Waste in the Food Industry*. V. Oreopoulou and R. Winfred (Eds.), ISEKI Series, Vol. 3, Springer, New York.
- CAST. (2003) Mycotoxins: Risks in Plant, Animal and Human Systems. Ames, IA: Council for Agricultural Science and Tecnology Task Force report n° 139.
- Chudoba P., Capdeville B. and, Chudoba J. (1992). Explanation of the biological meaning of the S0/X0 ratio in batch cultivation. *Wat. Sci. Tech.* 26, 743-751.
- OECD. (1984). OECD guideline for testing of chemicals. 302A: Inherent biodegradability: modified SCAS test. OECD, Paris.
- OECD. (1992). OECD guideline for testing of chemicals. 301: Ready biodegradability. OECD, Paris.
- OECD. (2001). OECD guideline for testing of chemicals. 303: Simulation test – Aerobic sewage treatment. 303A: Activated sludge units. OECD, Paris.
- Painter H. A. (1995). Detailed review paper on biodegradability testing. Environment monograph no. 98. Environment Directorate, OECD, Paris.
- van der Merwe K. J., Steyn P. S. and Fourie L. (1965). Mycotoxins. Part II. The constitution of ochratoxin A, B, and C, metabolites of *Aspergillus ochraceus* Wilh. *J. Chem. Soc.*, 7083-7088.
- Vishniac W. and Santer M. (1975). The Thiobacilli. *Bacteriol Ver.* 21, 195–213.