

Removal of Ochratoxin A in *Saccharomyces cerevisiae* Liquid Cultures

E. Bizaj¹, J. Mavri¹, F. Čuš² and A. Raspor^{1*}

(1) Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000, Ljubljana, Slovenia

(2) Agricultural Institute of Slovenia, Hacquetova 17, 1000, Ljubljana, Slovenia

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The capacity for removal of ochratoxin A (OTA) during alcoholic fermentation was evaluated in batch systems with one commercial strain and one wild strain of *Saccharomyces cerevisiae*. Batch alcoholic fermentations were carried out in yeast extract-malt extract broth (YM) medium, with 18.0% glucose and OTA added to final concentrations of 3.48 and 4.95 ng/mL respectively. The removal capacity of each yeast strain was examined after completion of fermentation in batch culture and after extended contact with yeast biomass. The removal capacity of the yeast strains was also examined in stationary phase cultures. Stationary phase yeasts were studied with biomass harvested from the stationary phase of anaerobic fermentation, by incubation in phosphate buffer, with the addition of 5.00 ng/mL of OTA. Removal studies with stationary phase cells were performed with viable and non-viable cells inactivated with Na-azide. The study showed that in growing phase cultures, OTA removal was significant only after extended contact with yeast biomass; up to 29.7% and 25.4% for wild yeast ZIM 1927 and commercial yeast Lalvin EC-1118 respectively, but not during alcoholic fermentation. In stationary phase cultures, viable and non-viable cells were not significantly different in OTA removal from the medium. This demonstrated that OTA was not metabolised, but possibly adsorbed by the yeast cells. The presence of OTA in synthetic media influenced yeast metabolism, causing the production of higher volatile acidity by 0.08 and 0.13 g/L for Lalvin EC-1118 and ZIM 1927 respectively, and lower concentrations of reducing sugar, by 0.32 g/L, but only for ZIM 1927.

Ochratoxin A is a strong nephrotoxic, carcinogenic, immunotoxic and teratogenic mycotoxin that can contaminate various foods and beverages, including wines. Its chemical structure consists of a chlorine-containing dihydroisocoumarin linked through the 7-carboxyl group to l- α -phenylalanine (Caridi, 2007).

Ochratoxin A (OTA) is a mycotoxin produced by two genera of fungi, *Aspergillus* and *Penicillium* (Mateo *et al.*, 2007). The development of the fungi-producing OTA essentially depends on climatic conditions, and it is found more frequently in regions with temperate and tropical climates (Zimmerli & Dick, 1996). The black aspergilli that infect grapes and produce OTA are present on berries from the early stages of development, thus an approach to prevent wine contamination involves identifying and controlling these fungi, especially *A. carbonarius* (Lo Curto *et al.*, 2004; Mateo *et al.*, 2007).

Because of strong toxicity, there is a big concern about the occurrence of ochratoxin A in many commodities, including feeds, foods and beverages (Marquardt & Frohlich, 1992). Since 1996, the presence of this toxin has been also reported in grapes and grape products such as grape juice (< 3–311 ng/L) and wine (< 3–388 ng/L) (Zimmerli & Dick, 1996). The presence of the fungal metabolite OTA in wine therefore represents a serious risk to the consumer's health. In fact, according to some studies, wine is considered the second major source of OTA intake after cereals (Mateo *et al.*, 2007). In this regard, red table wine samples have shown to have higher OTA concentrations than rose and white wine samples, but the highest concentrations have been found in dessert wines (Chulze *et al.*, 2006; Mateo *et al.*, 2007).

The EU has established a maximum allowable OTA concentration of 2 ppb for wines, starting from the vintage of 2005 (EC, 2005), reserving the possibility of lowering this limit following new technological and toxicological research.

In wine production, much OTA is removed in the solid-liquid separation stages during pressing in vinification, when the wine or juice is separated from the skins (Leong *et al.*, 2006). It has been reported that 50 to 80% of the total OTA content originally present in the crushed grapes is bound to the discarded skins and seeds. Various procedures have been developed to remove mycotoxins using yeasts, yeast cell walls or yeast cell wall extracts (Gambutti *et al.*, 2005; Ratola *et al.*, 2005; Caridi, 2007; Fernandes *et al.*, 2007; Hocking *et al.*, 2007). OTA is thought not to be degraded (biologically transformed) by yeasts during primary alcoholic fermentation; rather, the toxin is adsorbed onto yeast mannoproteins, but these vary in their adsorptive capacity. However, there is no firm consensus about the proposed mechanism involved in the removal of OTA (Bejaoui *et al.*, 2004; Caridi, 2006; Cecchini *et al.*, 2006; Hocking *et al.*, 2007). A great diversity has been observed among yeasts for their parietal adsorption activity: the outermost layer of the cell wall has a chemical composition that varies notably from yeast to yeast, and adsorption is very strain dependent. In the pH range of wine, mannoproteins carry negative charges and, as a consequence, they may establish electrostatic and ionic interactions with the other components of the wine (Huwig *et al.*, 2001; Caridi, 2006; Caridi, 2007).

The goal of our study was to find the potential of a *Saccharomyces cerevisiae* commercial yeast strain and a strain isolated from a

* Corresponding author: e-mail: peter.raspor@bf.uni-lj.si

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spontaneous fermentation to reduce the concentration of OTA added to a liquid synthetic medium, and to determine the influence of OTA at high concentrations on fermenting yeast strains.

MATERIALS AND METHODS

Yeast strains

The yeast strains used in this study were a commercially available *S. cerevisiae* Lalvin EC-1118 (Lallemand, Canada) (as active dry yeast) and the yeast strain *S. cerevisiae* ZIM 1927, previously isolated from a spontaneous fermentation of a cv. Malvasia grape must in 2001 and obtained from the culture collection of industrial microorganisms, University of Ljubljana, Slovenia. The first strain was rehydrated as described by the producer (Lallemand, Canada), and then cultured in the yeast extract-malt extract (YM) broth (0.3% yeast extract; 0.3% malt extract; 0.5% peptone; obtained from Biolife, Italy) and 18.0% glucose (for microbiology, Merck, Germany) at 28°C for 24 h, with rotary shaking at 190 rpm. The *S. cerevisiae* ZIM 1927 was a three-day-old culture, maintained on YPD agar (YPD Broth, Oxoid, England), and cultured in YM medium as described for Lalvin EC-1118.

Cultivation and medium

The alcoholic fermentations were carried out in anaerobic conditions, following the methodology used by Čuš and Raspor (2008). The cultivations were performed in liquid YM with 18.0% glucose at pH 6.37, previously sterilised by membrane filtration (0.20 µm, Sartorius, Germany). The medium, in a 300 ml fermentor, contained i) 280 mL of YM (control) and ii) 280 mL of YM spiked with OTA (Sigma-Aldrich, USA). The concentrations of OTA in the media, determined at time 0 days (Table 1), were 3.48 ng/mL and 4.95 ng/mL for ZIM 1927 and Lalvin 1118-EC respectively. The fermentations were performed in duplicate.

Culture conditions and sampling

The inocula were prepared as described above and added directly to the fermentors, to give a final concentration of 1×10^6 CFU/mL in the medium. The kinetics of fermentation were followed by CO₂ release measurement (weight loss). The fermentations were carried out at 20°C. The samplings for determination of the OTA concentration in the media were done at three stages: at time 0, at the end of the fermentation (less than 2 g/L of resting reducing sugars) and after seven days of contact with yeast biomass. After seven days of biomass-media contact, yeast biomass was also collected for OTA determination in the biomass and the fermented media. The latter were also analysed for volatile acidity and concentration of reducing sugars. Biomass was collected by centrifugation; three times at 11 200 x g for 10 min to remove supernatant.

Biomass recovery for stationary test

For the stationary test, the biomass produced during fermentations under anaerobic conditions (Čuš & Raspor, 2008) in liquid YM, with 18.0% of glucose at pH 6.37, was recovered by centrifugation (10 min, 11 200 x g) and washed three times (10 min, 11 200 x g) with phosphate buffer (pH 6.37, sterilised by membrane filtration (0.20 µm, Sartorius, Germany)). Finally, the cells were resuspended in the phosphate buffer to give a final concentration of 0.44 g/mL.

Stationary tests for biomass reduction of ochratoxin A

The test was performed in two replicates in tubes containing 5 mL of assay solution, consisting of 4.890 mL of phosphate buffer (pH

TABLE 1

Removal of OTA in YM media (18.0% glucose), pH 6.37 during stages of fermentation for *S. cerevisiae* yeast strains ZIM 1927 and Lalvin EC-1118 at 20°C. Data reported are average values of two independent experiments carried out under identical conditions.

Strain / fermentation stage		
Lalvin EC-1118	Concentration of OTA in medium (ng/ml)	% OTA in medium
Start fermentation	4.95 ^a	100.0
End fermentation	4.82 ^a	97.4
7 days media-biomass contact	3.69 ^b	74.6
ZIM 1927		
Start fermentation	3.48 ^c	100.0
End fermentation	3.41 ^c	98.0
7 days media-biomass contact	2.46 ^d	70.7

Start fermentation T = 0 days.

End fermentation: < 2 g/L glucose.

7 days after fermentation (daily mixing).

a,b,c : significant difference at $P \leq 0.05$.

6.37), with 0.010 mL of stock solution of OTA (Sigma-Aldrich, USA) added to give a final concentration of 5.00 ng/mL at 20°C. To each tube, 0.1 ml of a yeast biomass suspension (viable or non-viable cells) in phosphate buffer was added to give a final concentration of 0.044 g/mL. Non-viable cells were prepared by the addition of Na-azide (Sigma-Aldrich, USA) to biomass suspended in phosphate buffer (0.025% final concentration in media) in order to exclude metabolic action and preserve the structural integrity of the cells (Bejaoui *et al.*, 2004; Nunez *et al.*, 2007). After seven days of contact between the biomass and the media (Martínez-Rodríguez *et al.*, 2001a,b; 2002; Nunez *et al.*, 2007), with or without agitation (rotary shaking of tubes at 190 min⁻¹), the solution was centrifuged and the non-adsorbed amounts of OTA in the medium were measured.

Extraction of OTA from yeast biomass and YM media

The extraction of OTA from yeast biomass and YM samples was performed using the organic extraction protocol for wine and grape samples, proposed by the manufacturer of the enzyme immunoassay kit used, I'screen OchrA (Tecna S.r.l., Trieste, Italy), which in our case was modified for 500 µL sample volumes. Briefly, 100 mg of sample wet biomass was first transferred in a 2 mL Eppendorf tube, washed two times with 1 mL of distilled water, centrifuged (5 min, 1 500 g) and resuspended in 400 µL of H₂O. In the case of YM media, 500 µL of sample was collected in the tube without further treatment. To each sample, 500 µL 1M HCl and 1 mL of dichloromethane were added and mixed at 1 200 rpm in a rotary shaker (TTS2, IKA, USA) for 15 min at room temperature. This was followed by centrifugation at 2 200 g for 15 min. Then, 500 µL of the lower dichloromethane phase was pipetted in another 2 mL Eppendorf tube and 250 µL of 130 mM NaHCO₃ (pH 8.1) was added. This was mixed again for 15 min at 1 200 rpm. Finally, samples were centrifuged at 2 200 g for 15 min, and 200 µL of the upper polar phase was taken and analysed with the immunoassay kit.

The yield of OTA extraction from yeast biomass, determined from a series of five independent extractions using 100 mg cell biomass spiked with 25 ng OTA, was $37.9 \pm 1.5\%$.

Determination of OTA residues

The determination of OTA residues in the extracts of YM medium and cell biomass was performed with the commercial kit L'screen Oehhra (Tecna S.r.l., Trieste, Italy), following the manufacturer's protocol.

Determination of reducing sugars and volatile acidity

After the cultivation, the reducing sugars and the volatile acidity in the medium were determined according to the accredited methods in the Central Laboratory of the Agricultural Institute of Slovenia. Reducing sugars were determined according to the modified Rebelein method: 10 mL of the alkaline copper salt solution, 5 mL of Segnet salt and 2 mL of sample were placed in a 300 mL Erlenmeyer flask. The mixture was boiled for exactly 45 seconds. The flask was cooled down immediately and 5 mL of 30% potassium iodide solution, 5 mL of 16% sulphuric acid and 5 mL of starch solution (10.0 g/L) were added. The mixture was titrated with 0.055 M sodium thiosulphate solution (to a yellow-cream colour). Blank titration was carried out, in which the 2.0 mL of sample was replaced with 2.0 mL of distilled water. The concentration of the reducing sugars in the sample was calculated according to the volume of sodium thiosulphate used in both titrations (EEC, 1990).

Volatile acidity was determined by titration of the distillate obtained by steam distillation. A total of 20 mL of sample, freed from carbon dioxide, was placed in the distillation flask and 0.5 g of tartaric acid was added. Distillation was done with steam distillation apparatus (Oenoextracteur Chenard). At least 250 mL of the distillate was collected. The distillate was titrated with 0.1 M sodium hydroxide solution, using phenolphthalein as an indicator. The volatile acidity expressed in grams of acetic acid per litre was calculated according to the volume of sodium hydroxide used in the titration (EEC, 1990).

Statistical analysis

The analysed data were studied by one-way ANOVA (Microsoft Office Excel 2003, USA). The statistical level of significance was set at $P \leq 0.05$. The means were compared with the Tukey test (T-test).

RESULTS AND DISCUSSION

OTA removal during alcoholic fermentation and yeast biomass contact

Fermentation kinetics (Fig. 1), which were followed by the daily release of CO₂ (weight loss), showed no significant differences between the controls and the fermentations with added OTA. After the completion of fermentation, the yeast biomass was maintained in contact with the media for seven days by daily mixing. During this time, the major proportion of mannose was released from the yeast cell walls into the media (Nunez *et al.*, 2007) due to accelerated autolysis triggered by the lack of nutrients (Martinez-Rodriguez *et al.*, 2001a; 2001b; 2002). However, during the alcoholic fermentation conducted by both yeast strains, no significant reduction was found in the concentration of OTA. The concentrations detected were 3.41 ng/mL and 4.82 ng/mL, for ZIM 1927 and Lalvin EC-1118 respectively (Table 1). On

the other hand, a significant reduction was observed after seven days of contact between the media and yeast biomass. The final concentrations detected in the media were 2.46 ng/mL and 3.69 ng/mL for ZIM 1927 and Lalvin EC-1118 respectively. Under the conditions used in our study, the two strains were unable to significantly reduce the concentration of OTA in synthetic media during the alcoholic fermentation phase. This result contrasts with that reported by Bejaoui *et al.* (2004), who used different conditions that have been found to affect OTA removal potential: pH, media and strains (Bejaoui *et al.*, 2004; Caridi, 2006; 2007; Cecchini *et al.*, 2006). In the post-fermentation period, 25.4% (Lalvin EC-1118) and 29.3% (ZIM 1927) reductions were achieved by the yeast strains. At the same time, although the initial concentration of OTA in the media was different for the two strains, the degree of removal induced by the two strains was not significantly different. These results suggest that the release of mannoproteins from cell walls after the completion of fermentation (Caridi, 2006; 2007; Nunez *et al.*, 2007) is important in the removal of OTA.

Concentration of OTA in wet yeast cell biomass

To determine the OTA removal potential of yeast biomass in the growth assay, the yeast biomass was recovered from the medium after the extended medium-biomass contact period (seven days) and the cell-associated OTA concentration was determined. For the ZIM 1927 strain, 15.92 ng OTA/100 mg of wet weight biomass was found, and 14.49 ng OTA/100 mg of wet weight biomass for the Lalvin EC-1118 strain. In spite of the different origins of these two strains, no significant difference was detected in OTA removal capacity, which was unexpected in the light of other reports (Bejaoui *et al.*, 2004; Caridi, 2006; 2007; Cecchini *et al.*, 2006).

OTA removal in yeast stationary phase biomass tests

The yeast stationary phase assays were performed to compare the OTA removal potential of viable and non-viable yeast biomass, and to determine whether agitation during biomass-medium contact (rotary shaking of tubes at 190 min⁻¹) affects the OTA removal as well. We tested fresh, washed biomass, in phosphate buffer (pH 6.37), to ensure that other compounds present in the cultivation media did not affect the reduction of OTA. The pH was the same as in growth assays.

In the results of others researchers (Bejaoui *et al.*, 2004; Moruno *et al.*, 2005), the biomass concentration in the assay media is critical when determining OTA removal potential, and we therefore used the same concentration (0.0088 g/mL) for both strains (media of wet weight biomass produced by the two yeasts in batch alcoholic fermentation carried out in YM medium with 18.0% glucose; results not shown). Independently of the adsorption assay type, significant removal of OTA was obtained (Table 2). The results show that, for both strains, there are no significant differences between viable and non-viable yeast biomass OTA removal when comparing within the same strain and the same treatment (agitated or not agitated) (Table 2). These results suggest strongly that OTA is not degraded by yeast metabolism and that adsorption of OTA is a likely mechanism to account for its reduction. As in the stationary stage of the growth assay, as well as in the stationary assays, the release of mannoproteins appeared to trigger OTA reduction (Nunez *et al.*, 2007) in both viable and non-viable biomass. The strain ZIM 1927 reduced OTA the most in the case of dead biomass when agitation was applied – by 54.4%. This was

statistically significantly different compared to the trials with the strain ZIM 1927 viable and non-viable biomass when agitation was not applied, when only 21.0 and 21.4% removal respectively was obtained. The trend of a higher degree of OTA removal in agitated assays when compared to non-agitated assays of the same strain and viability is strongly present (Table 2). However, this is confirmed statistically only in ZIM1927 non-viable biomass assays. For strain Lalvin-1118-EC there were not statistically significant differences between the viable and non-viable yeast biomasses. Along with the confirmation that the mechanism of OTA removal by these two *S. cerevisiae* yeast strains involves adsorption, the extent of adsorption is perhaps lower than it

might be at a lower pH (Bejaoui *et al.*, 2004; Ringot *et al.*, 2005). Adsorption is affected by pH because of different adsorption mechanisms used by different microorganisms, and because of cell wall composition (Huwig *et al.*, 2001).

Volatile acidity and reducing sugars

The concentrations of volatile acids and reducing sugars were determined in the culture media after the seven-day period of media-biomass contact (Table 3). It can be seen that, for both strains, OTA addition to the media caused significantly higher production of volatile acidity. The concentrations produced in batch fermentations to which OTA was added initially were higher by 0.08 and 0.13 g/L for the strains Lalvin EC-1118 and

TABLE 2

Removal of OTA in phosphate buffer pH 6.37 in stationary tests, after treatments with different types of yeast cell biomass for seven days at 20°C. Data reported are average values of two independent experiments carried out under identical conditions.

Treatment in phosphate buffer pH = 6.37	OTA ng/mL	% of OTA
ZIM1927 Non-viable cells	3.93 ^b	78.6
ZIM1927 Non-viable (R)	2.28 ^c	45.6
ZIM1927 Viable cells	3.95 ^b	79.0
ZIM1927 Viable cells (R)	3.10 ^{b,c}	62.0
EC-1118 Non-viable cells	4.09 ^b	81.8
EC-1118 Non-viable cells (R)	3.64 ^b	72.8
EC-1118 Viable cells	4.33 ^b	86.6
EC-1118 Viable cells (R)	3.90 ^b	78.0

Concentration of dead or live yeast biomass added in tubes was 0.0088 g/ml. Initial concentration of OTA (ochratoxin A) in media 5.00^a ng/mL. R: Agitated: (rotary mixing of tubes at 190 min⁻¹). a,b,c: significant difference $P \leq 0.05$.

TABLE 3

Concentration of reducing sugar after 29 days of alcoholic fermentations with OTA conducted in YM with 18.0% glucose and pH 6.37 for the *Saccharomyces cerevisiae* strains ZIM 1927 and Lalvin EC-1118 at 20°C. Data reported are average values of two independent experiments carried out under identical conditions.

Sample	Concentration of reducing sugars (g/L)	Concentration of volatile acidity (g/L)
EC-1118	1.50 ^a	0.45 ^a
EC-1118 OTA	1.00 ^a	0.53 ^b
ZIM 1927	0.40 ^b	0.48 ^a
ZIM 1927 OTA	0.08 ^c	0.61 ^c

Control (ZIM 1927, 1118EC), OTA was not added. OTA was added to the medium (ZIM 1927 OTA, 1118 EC OTA). a,b,c, significant difference at $P \leq 0.05$. Mean values with the same letter in the same column do not differ significantly $P \leq 0.05$.

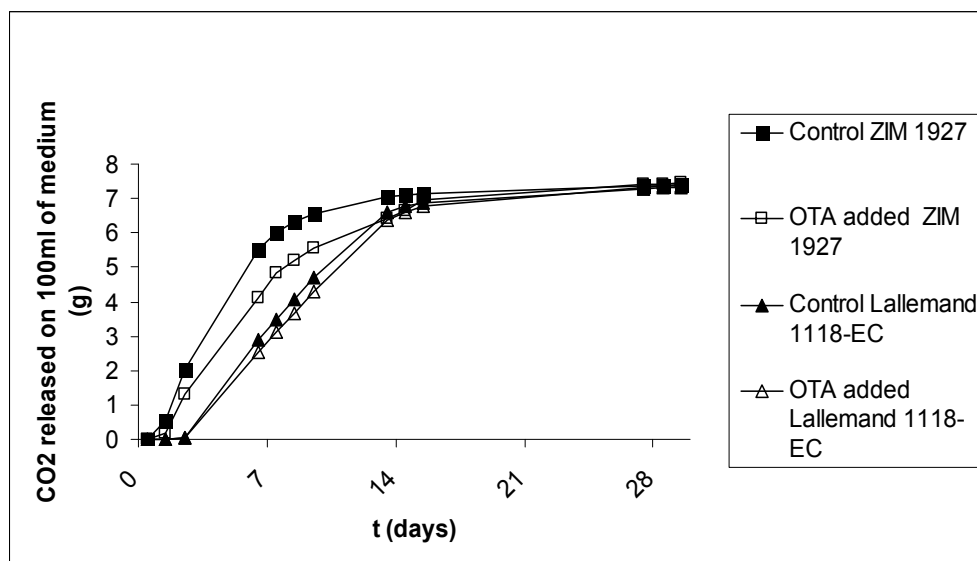


FIGURE 1

Growth of ZIM 1927 and Lalvin 1118EC (Lallemard) in YM medium (glucose: 18.0%; pH 6,37; incubation at 20°C in semianaerobiosis) in the presence of OTA formulations; 4.95 ng/mL for Lalvin 1118-EC and 3.48 ng/mL for ZIM 1927 or in the absence of OTA (control). Data reported are average values of two independent experiments carried out under identical conditions.

ZIM 1927 respectively, in comparison to fermentations where OTA was not added (controls). The production of volatile acidity was significantly higher for strain ZIM 1927, suggesting that this strain was more sensitive than Lalvin EC-1118 to the presence of OTA. This is also suggested by the fact that, even although the initial concentration of OTA added was higher for Lalvin EC-1118, by 1.47 ng/mL in comparison to ZIM 1927, the former is producing 0.08 g/L less volatile acidity. These data show that the concentrations of OTA used in these experiments were sufficient to affect the production of volatile acidity by wine strains of *S. cerevisiae* in synthetic media.

Contrary to the production of volatile acidity, OTA did not negatively affect the fermentation capacity of these yeast strains or, consequently, the concentration of reducing sugars in the media. Independently of OTA addition, Lalvin EC-1118 shows lower fermentation capacity in comparison to ZIM 1927, and there were no significant differences whether OTA was added to the former or not. On the other hand, the addition of OTA to fermentations with the ZIM 1927 strain resulted in a lower concentration of reducing sugars, and this was significantly lower (0.08 g/L) in comparison to the concentration detected in the control fermentation (0.40 g/L).

CONCLUSIONS

The aim of our study was to investigate the potential of viable and non-viable *S. cerevisiae* yeast biomass to reduce the concentration of OTA in synthetic media. The two genetically different wine yeast strains studied, Lalvin EC-1118, which is widely used commercially, and ZIM 1927, a laboratory strain previously isolated from a spontaneous fermentation of grape must, were able to significantly lower OTA concentrations in synthetic media. The evaluation of the adsorption capacity of yeast biomasses showed no significant differences between the commercial and wild strains tested. In our study we could not observe the removal of OTA during alcoholic fermentation, and significant removal of OTA was found only after the extended medium-biomass contact period (seven days), possibly as a consequence of yeast autolysis and the action of mannoproteins. Our results demonstrate that non-viable yeast cells, that is cells that are inactivated by an agent that does not affect cell wall integrity (in contrast to heat or acid treatment), do not show significantly different potential to remove OTA when compared to viable yeast cells in the stationary phase. These results suggest that OTA is not metabolised by the yeast. Moreover, this indicates that removal only involves adsorption processes mediated by yeast constituents.

Finally, the presence of OTA in synthetic media was found to influence fermentation capacity and the production of volatile acidity, which indicates that OTA can influence selected metabolic processes in synthetic media.

Further work will be needed to demonstrate that yeasts interact similarly with OTA in grape must.

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