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Abstract

This work aims to investigate the performance of 20 strains of *Saccharomyces sensu stricto* to remove ochratoxin A (OTA) during vinification. Each strain was inoculated in triplicate in 10mL of white must with a natural OTA content of 1.58 ng/mL, and again in the same must with the addition of OTA, to reach a total content of 7.63 ng of OTA/mL. This microvinification trial was performed at 25 °C for 90 days. The OTA content and the ethanol content of the wines were assayed; the OTA content of the lees was also analysed separately. The OTA content in wines produced from the naturally OTA-containing must varied from 0.143 to 0.950 ng of OTA/mL (mean 0.498 ng of OTA/mL). The OTA content in wines obtained from the must with the addition of OTA varied from 1.270 to 2.448 ng of OTA/mL (mean 1.661 ng of OTA/mL). The OTA content in lees varied from 1.537 to 7.456 ng of OTA/mg of biomass (wet weight). Interestingly, this last result indicates that OTA-removal from grape must was probably carried out by the yeast cell wall, acting like a sponge. A role of the parietal yeast mannoproteins in OTA adsorption during winemaking was hypothesised and the implications of these results in the winemaking of OTA-contaminated musts were discussed.

Keywords: Saccharomyces sensu stricto; Adsorption; Lees; Ochratoxin A removal; Parietal yeast mannoproteins; Vinification

1. Introduction

Ochratoxin A (OTA) is a dangerous fungal secondary metabolite; this mycotoxin frequently occurs in various foods and beverages and is produced by a few species belonging to the *Aspergillus* and *Penicillium* genera.
Due to the high toxicity of mycotoxins, many methods to control their effects have been proposed. One of the most effective approaches is the use of specific materials that adsorb mycotoxins, thus avoiding or limiting their bioavailability [1]. Increasing interest has been recently generated by the possibility of using microbiological-binding agents to remove mycotoxins [2,3].
Since 1996, OTA has been reported in grapes, grape juices and wines [4]. The contribution of wine to mean daily OTA intake cannot be considered negligible. Indeed, considering available data regarding the presence of OTA in alcoholic beverages, wine alone could supply an average drinker with an important part of the maximum total daily OTA intake recommended by the Scientific Committee on Food of the European Commission [5]. In alcoholic beverages, OTA is formed prior to alcoholic fermentation, during which, however, it is partially removed or degraded [6]. Interestingly, this decrease in OTA is strain-dependent [7].
Different decontamination procedures using *Saccharomyces* strains have recently been proposed for OTA-removal [3,8,9,10], but no study has been carried out to select starter yeasts able to remove OTA during alcoholic fermentation. The idea of screening wine yeasts started from the early observation that OTA added to wort at a level of 0.19 μg/mL and fermented for up to 8 days by three strains of *Saccharomyces cerevisiae* decreased up to 21% with one strain [7].
In a previous study which tested the ability of twenty selected wine yeasts to remove OTA from a physiological sterile saline solution, we observed a wide biodiversity among strains [10].
The present work aims to investigate the performance of the same strains during alcoholic fermentation.

2. Material and methods

2.1. Strains and methodology

Twenty strains of *Saccharomyces sensu stricto*, previously selected for winemaking and studied *in vitro* for OTA removing capacity [10] were employed. Each yeast strain was inoculated in triplicate in test tubes containing 10mL of must obtained from Calabrian white grapes with a natural OTA content of 1.58 ng/mL, and again in triplicate using the same must with the addition of OTA, to reach a total content of 7.63 ng/mL. This microvinification trial was performed at 25 °C for 90 days and then wines and lees were analysed. The toxin content and the ethanol content of the wines were assayed; the toxin content of the lees was also analysed separately.

2.2. Determination of the ethanol content in wines

The samples were filtered through a 0.45mm mesh before a gas-chromatographic analysis performed on a Varian model Star 3400CX instrument equipped with a DBWAX capillary column (length 30 m, internal diameter 0.25 mm, film

thickness 0.25 mm). Operating conditions were a helium flow rate of 0.7 mL/min, a FID detector at 260 °C, a split–splitless injector at 220 °C, and an injection volume of 1 mL. The temperature programme of the column was 5 min at 50 °C and a subsequent increase to 150° at 10 °C/min and 3 min at 150 °C. The ethanol was identified by comparing the retention time of a standard (purity >99%) purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Determination of the OTA content in wines and in lees

The HPLC analyses were performed using LC-10AD pumps and a fluorescence detector, model RF-10Axl (Shimadzu, Japan), set to an excitation wavelength of 333 nm and emission wavelength of 460 nm. Data acquisition and handling were made by a system control SLC10A with software VP5 (Shimadzu, Japan). A Jupiter (Phenomenex, USA) C18 (250mm×4.6 mm, 5μm) column was used. The HPLC conditions were set up using elution at a constant flow of 1 mL/min andCH_3CN (1% acetic acid) – H_2O (1% acetic acid) (50:50 v/v) as the starting eluent system. The starting ratio was linearly modified to 100% CH_3CN in 15 min. From the 15th to 18th min the pumps were taken back to starting conditions and then the isocratic conditions were taken for 5 min. Eluent was freshly prepared and filtered (0.22μm) before use. All samples were filtered through a 0.22μm syringe filter (Millipore, Bedford, MA, USA) prior to injection (20μL) into the HPLC column by 250mL syringe (Hamilton, Switzerland). Mycotoxin identification was performed by comparing retention times and UV spectra of purified samples to a pure OTA standard. A further confirmation was performed by co-injecting samples together with an OTA standard solution. The average retention time for OTA (4.5 min, R.S.D. 1.5%) was obtained with 10 consecutive injections of the same OTA working solution within the same day.
This retention time enhanced the chromatographic resolution of the OTA peak from other matrix interferences. The calculated instrumental detection limit and quantification limit for OTA under these conditions were 0.2 (10 ng/L, S/N 3) and 2 pg (100 ng/L), respectively. Mycotoxin quantification was carried out by comparing peak areas of investigated samples to the calibration curve, ranging from 0.1 to 100μg/L, of authentic OTA standards. All the analysed samples were prepurified prior to injection by Ochraprep immunoaffinity columns (RBiopharm Rhône, Glasgow, Scotland) and eluted at a flow rate of 1–2 drops/s. The column was washed with 20 ml of water and then eluted with 1.5mL of methanol (acetic acid 2%) and 1.5mL of pure water. The difference between the OTA content of wine and grape must was divided by the OTA content of the grape must and expressed as percentage.
The OTA content in lees was determined as follows: cells were harvested by centrifugation (centrifuge Juan model CR3i), sonicated in saline solution (0.85%, w/v, NaCl) for 30 min and washed twice with saline solution (4000 rpm for 10 min at 4 °C). The pellet was suspended in 3mL of saline solution, 3mL of ethyl acetate were added and, after mixing and centrifugation (4000 rpm for 10 min at 4 °C), 1mL of the top phase was evaporated by rotavapor (system Juan model RC60), re-suspended in 1mL of methanol and analysed by injecting 60μL into the HPLC column by 250mL syringe (Hamilton, Switzerland). All the analytical data were elaborated by ANOVA analysis.

3. Results

The results of the analysis of the wines produced from the naturally OTA-containing must are reported in Table 1. The mean ethanol content was 6.53% (v/v) with a minimum of 4.44 and a maximum of 9.58% (v/v). The OTA content, from 1.58 ng of OTA/mL in the grape must, was reduced in the wines to a mean value of 0.50 ng/mL with a minimum of 0.14 and a maximum of 0.95, depending on the strain used. There are significant differences ($P < 0.05$) among the yeast strains for the residual OTA values; strains Sc254 and Sc2659 exhibited the highest capacity to remove OTA, while strains Sc1661 and Sc2717 showed the lowest capacity. The other 16 strains exhibited intermediate abilities. However, all the tested strains were able to noticeably and significantly reduce the initial OTA content, as the difference between the OTA content of the grape must and of the wines was statistically significant for all the yeasts (ANOVA results not shown).
The results of the analysis on the wines produced from the must with the addition of OTA are reported in Table 2. The mean ethanol content was 7.75% (v/v) with a minimum of 5.57 and a maximum of 11.94% (v/v). The OTA content, from 7.63 ng of OTA/mL in the grape must, was reduced in the wines to a mean value of 1.66 ng/mL with a minimum of 1.27 and a maximum of 2.45, depending on the strain. There were highly significant differences ($P < 0.01$) between the OTA content of the grape must and of the wines for all the yeasts. All the strains were able to noticeably and significantly reduce the initial OTA content, with no significant difference in the behaviour of each strain.
Fig. 1 shows the percentage of removed OTA in wines obtained from the naturally OTA-containing grape must. The value had a mean of 68.45%, in a wide range, from 39.81% (strain Sc1661) to 90.95% (strain Sc254).
Fig. 2 shows the percentage of removed OTA in wines obtained from the grape must with the addition of OTA. The value had a mean of 78.22% with a much narrower range, from 67.89% (strain Sc708) to 83.34% (strain 12233).
Analysis of UV coupled to fluorescence HPLC chromatograms (results not shown) showed no peaks other than OTA, thus indicating that no products of OTA breakdown were released in wines. Consequently, we may suppose that OTA was removed by *Saccharomyces sensu stricto* in a cell-binding phenomenon, according to Ref. [3].
The results of the OTA analysis on the lees from the naturally OTA-containing grape must showed a mean OTA content of 4.80 ng of OTA/mg of biomass (wet weight) with a minimum of 1.54 and a maximum of 6.31. The results of the OTA analysis on the lees from the grape must with the addition of OTA showed a mean OTA content of 4.53 ng of OTA/mg of biomass (wet weight) with a minimum of 2.29 and a maximum of 7.46. These extremely interesting results indicate that OTA-removal from grape must was probably carried out by the yeast cell wall, acting like a sponge. The concentration

of the mycotoxin in the lees was up to 18 times greater than the residual content in the corresponding wines. The quantity of ethanol produced is an indicator of yeast activity and, consequently, of the total yeast biomass at the end of fermentation. Since a higher ethanol content is not a reliable indicator of a yeast's ability to adsorb OTA, we may conclude that this adsorption depends more on the strain of yeast used than on the biomass produced.

4. Discussion

The yeast cell wall is made up of two principal constituents: β-glucans and mannoproteins. Mannoproteins constitute 25–50% of the cell wall of *Saccharomyces*, and their degree of glycosylation is variable, as in some cases they can contain up to 90% mannose, with 10% of peptides being hypermannosylated. Mannoproteins are partially water-soluble components that are released during and, above all, at the end of alcoholic fermentation. The mannoproteins located in the outermost layer of the yeast cell wall give this structure its active properties and have an important role in controlling the wall's porosity [11–13], thereby regulating leakage of proteins from the periplasmic space and entrance of macromolecules from the environment. At different pH values, the electrical charge of the parietal yeast mannoproteins is modified; in the pH range of wine, mannoproteins carry negative charges and, as a consequence, they may establish electrostatic and ionic interactions with the other wine components [14]. In contrast to mannoproteins, in the pH range of wine, phenolic compounds carry no or negligible negative charges, so that electrostatic and ionic forces are not determinant to their physico-chemical reactivity [15]. In several yeasts, including the genus *Saccharomyces*, the glycan portion of mannoproteins is composed not only of neutral oligosaccharides containing mannose and *N*-acetylglucosamine, but also of acidic oligosaccharides containing mannosylphosphate [16], in quantities which vary from strain to strain. This structural variability may explain the differences in the binding activity of wine yeasts towards phenolic compounds [17] and OTA [10]. Mannoproteins could be implicated in the OTA adsorption from contaminated grape musts, because of their ability to bind mycotoxins attributed to modified mannanoligosaccharide derived from the cell wall of *Saccharomyces* [18]. Moreover, the strains' ability to bind OTA in physiological sterile saline [10] is quite different to this ability in wine. This could be due to various factors:

- The mannosylphosphate residues confer a net negative charge to the cell wall, thus changing the properties and environment of the cell surface; this negative charge is probably present both at the pH of the wine and at neutral pH. Since OTA is a weak acid, it presumably remains in a non-charged state at the pH of the wine; whereas, at neutral pH OTA shows a net negative charge. Consequently, at neutral pH the strains of *Saccharomyces* that possess mannosylphosphate-free mannoproteins, i.e. neutral oligosaccharides, should be more able to adsorb OTA. Both the complexity and thepHof the wine make the behaviour of the parietal yeast mannoproteins difficult to predict.
- The composition of the cell wall is strongly influenced by nutritive conditions; the proportion of glucan in the cell wall increases according to the amount of sugar in the culture medium [19].
- In wine, mannoproteins adsorb not only OTA, but, at different levels of adsorption, also other components, such as phenolic compounds.

5. Perspectives

The removal of OTA from wine is a pressing problem and many materials have been tested for this purpose [20]. The need for an efficient removal agent appears to be of particular importance in consideration of the decision of the European Community that has fixed a maximum limit of 2.0 ppb of OTA [21] for wines produced from the 2005 harvest onwards. In this context yeasts represent a very promising biomaterial, also considering their role in the vinification process. Our results demonstrate the possibility of greatly reducing the OTA content of grape must with expressly selected wine yeasts, used like a sponge, sequestering OTA during winemaking. Further research will be carried out to validate, on a winery-scale, the ability of the best strains of *Saccharomyces sensu stricto* to remove OTA during winemaking and to study the adsorbing behaviour of neutral and acid mannoproteins on different compounds present in wine.

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Table 1. Ethanol (%, v/v) and residual OTA (ng/mL) values in wines obtained with 20 different wine yeasts using a naturally OTA-containing grape must (90 days of fermentation)

Strain	Ethanol	Residual OTA
Grape must	0	1.58 ± 0.33 a
1042	5.82 ± 0.91	0.66 ± 0.22 bc
12233	4.44 ± 0.57	0.24 ± 0.30 bc
Sc45	6.15 ± 0.07	0.29 ± 0.14 bc
Sc226	6.60 ± 2.51	0.87 ± 0.10 bc
Sc254	5.25 ± 0.61	0.14 ± 0.12 c
Sc560	9.58 ± 3.52	0.31 ± 0.16 bc
Sc708	5.65 ± 0.52	0.44 ± 0.26 bc
Sc1304	6.14 ± 3.39	0.55 ± 0.39 bc
Sc1483	5.95 ± 1.81	0.48 ± 0.12 bc
Sc1661	4.85 ± 0.82	0.95 ± 0.39 b
Sc1766	7.10 ± 3.06	0.61 ± 0.10 bc
Sc1864	6.87 ± 1.33	0.53 ± 0.14 bc
Sc2489	8.79 ± 0.61	0.79 ± 0.45 bc
Sc2621	6.63 ± 3.21	0.46 ± 0.11 bc
Sc2640	6.40 ± 3.50	0.39 ± 0.25 bc
Sc2659	6.12 ± 1.20	0.19 ± 0.20 c
Sc2717	7.81 ± 1.41	0.93 ± 0.20 b
TT77	5.17 ± 0.55	0.39 ± 0.17 bc
TT173	7.43 ± 2.25	0.22 ± 0.21 bc
TT254	7.86 ± 1.47	0.53 ± 0.25 bc
Mean	6.53 ± 1.67	0.50 ± 0.21

Values with different letters differ at $P < 0.05$.

Table 2. Ethanol (%, v/v) and residual OTA (ng/mL) values in wines obtained with 20 different wine yeasts using grape must with the addition of OTA (90 days of fermentation)

Strain	Ethanol	Residual OTA
Grape must	0	7.63 ± 0.62 a
1042	6.86 ± 1.07	1.73 ± 0.47 b
12233	7.09 ± 2.76	1.27 ± 0.28 b
Sc45	8.32 ± 4.04	1.39 ± 0.53 b
Sc226	7.02 ± 3.37	2.05 ± 0.18 b
Sc254	6.82 ± 3.82	1.37 ± 0.31 b
Sc560	8.79 ± 4.17	1.43 ± 0.44 b
Sc708	11.94 ± 2.89	2.45 ± 0.95 b
Sc1304	5.57 ± 0.97	1.83 ± 0.19 b
Sc1483	7.32 ± 0.73	1.52 ± 0.34 b
Sc1661	8.71 ± 3.23	1.83 ± 0.24 b
Sc1766	9.80 ± 1.87	1.70 ± 0.29 b
Sc1864	8.21 ± 3.81	1.56 ± 0.26 b
Sc2489	7.51 ± 2.90	1.43 ± 0.05 b
Sc2621	6.28 ± 1.53	1.57 ± 0.35 b
Sc2640	7.72 ± 2.28	1.89 ± 0.16 b
Sc2659	10.04 ± 3.59	1.35 ± 0.20 b
Sc2717	9.35 ± 3.36	2.03 ± 0.61 b
TT77	5.87 ± 0.33	1.64 ± 0.34 b
TT173	6.12 ± 0.79	1.54 ± 0.43 b
TT254	5.62 ± 1.04	1.63 ± 0.27 b
Mean	7.75 ± 2.43	1.66 ± 0.34

Values with different letters differ at $P < 0.01$.

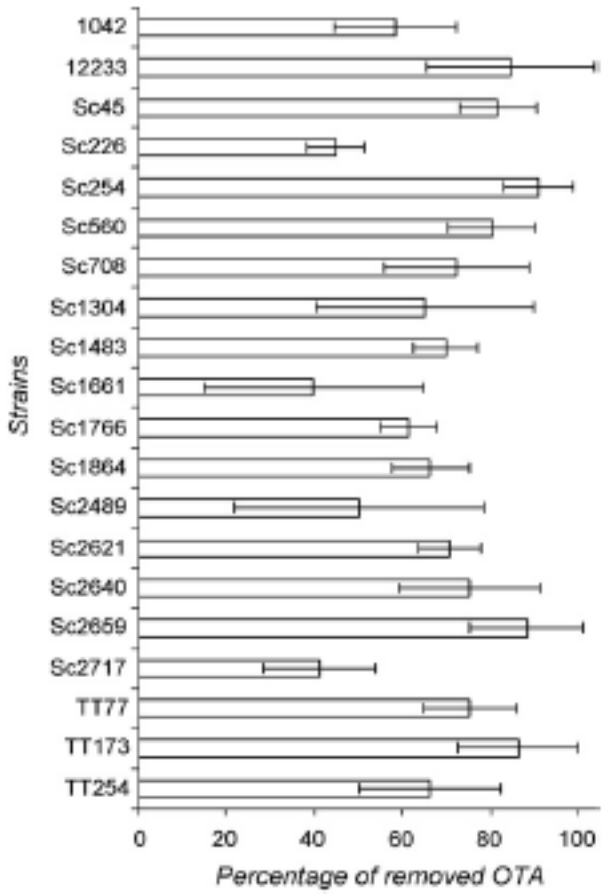

Fig. 1. Removal of OTA (%) in wines obtained with 20 different wine yeasts, using grape must with a natural OTA content of 1.58 ng/mL, after 90 days of fermentation.

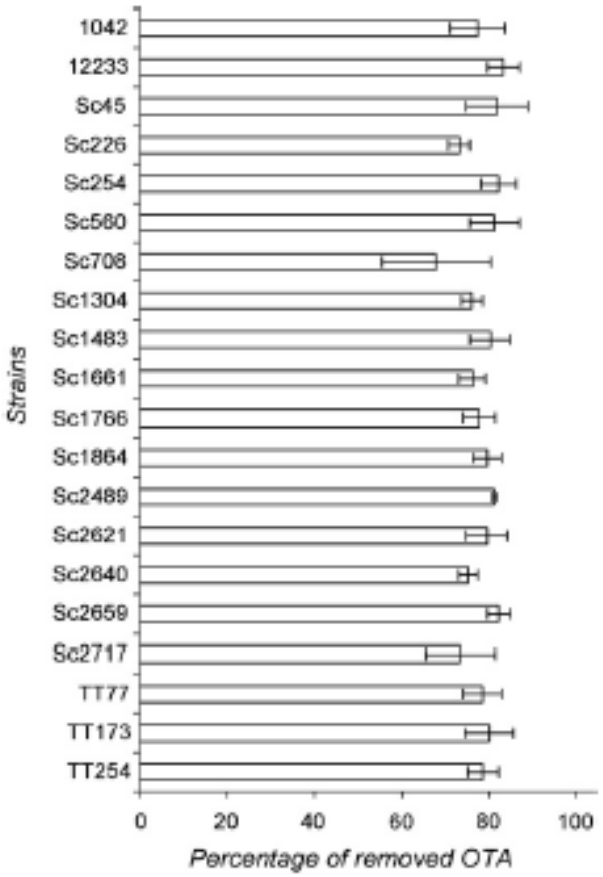

Fig. 2. Removal of OTA (%) in wines obtained with 20 different wine yeasts, using grape must with the addition of OTA to reach a total content of 7.63 ng of OTA/mL, after 90 days of fermentation.