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ORIGINAL PAPER

Removal of ochratoxin A by wine *Saccharomyces cerevisiae* strains

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Abstract The aim of this work was to examine two wine strains of *Saccharomyces cerevisiae* (Syrena LOCK 0201 and Malaga LOCK 0173 strains) and thermally inactivated biomass of bakery yeast (BS strain) for their ability to remove ochratoxin A (OTA) from model YPG, white grape GM, and blackcurrant BM media. The media was initially contaminated by 1 μg/mL OTA. The influence of OTA on yeast growth parameters, kinetic of fermentation, and amount of ethanol, glycerol, and acids were determined. It was found that both yeast strains were able to decrease the toxin amount in YPG, GM, and BM media. Strain Malaga LOCK 0173 was able to remove 82.8 and 10.7 % ochratoxin A from grape and blackcurrant medium, respectively. In case of Syrena LOCK 0201 strain, the OTA reduction was higher: 85.1 % for grape and 65.2 % for blackcurrant media. From 54.1 to 64.4 % of initial ochratoxin A concentration was removed after the contaminated wine treatment by thermally inactivated baker's yeast strain (BS) cells. The elongation of lag phase in contaminated YPG medium compared on toxin-free medium was noted. In white grape and blackcurrant medium, the differences between the final cell number, fermentation rate, moreover the ethanol, glycerol, and acids production in the medium with OTA and the control were not statistically significant. The results showed that the application of selected strains of yeasts in winemaking involving raw material contaminated with OTA might reduce the toxin contamination as well as the health risk related to human exposure to this toxin. Moreover, the application of heat-inactivated yeast's biomass for toxin adsorption gives new possibilities in oenology.

Keywords Ochratoxin A · *Saccharomyces cerevisiae* · Detoxification · Fermentation · Adsorption

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Introduction

Ochratoxin A is very widely detected in raw material and food products such as cereals, bread, coffee, dried wine fruits, as well as in beverages, for example beer, grape juice, and wine [1–7]. This toxin is produced by numerous fungal species belonging to the genera *Aspergillus* and *Penicillium*. The primary OTA-producing species associated with grapes belong to *Aspergillus* genera, section *Nigri* (black aspergilla), and including *Aspergillus niger* var. *niger*, *A. niger* var. *awamori*, *A. carbonarius*, *A. foetidus*, *A. biseriate*, and *A. uniserate* [8, 9]. Factors affecting wine contamination by ochratoxin A and their occurrence in red, rose, white, and special wines are reviewed in several articles [5–7, 10].

The presence of OTA in wine is mainly a result of fungal contamination on grapes both at pre and postharvest. This toxin is more prevalent in wines originating from the Mediterranean basin, less from Australia and South America [2, 4, 11, 12]. There is no information about the distribution of OTA in fruit alcohol beverages, which are popular in some countries, for example in Poland. Ochratoxin A contamination of commodities is a serious health problem due to their carcinogenic, nephrotoxic, genotoxic, and teratogenic properties [13]. In order to protect consumers, the EU Committee has established the maximum OTA levels for wines and musts at 2 μg/L [14].

In order to minimize the risk of OTA contamination, the preventive actions including the selection and proper

storage of raw material are used. Such actions are not always completely effective. Several possibilities are available as for the detoxification of mycotoxins, including physical and chemical methods [15–18].

Few of these have practical application, for example various inorganic adsorbent such as aluminosilicates, zeolites, bentonites, clays, and activated carbon. The using of these agents has a lot of disadvantages; in many cases, they decrease the nutritive value and organoleptic properties or significantly increase the cost of food and feed production [17, 18]. Recently, an increase in the research connected with mycotoxin detoxification by microorganisms has been observed. Several studies showed that some bacteria, moulds, and yeasts, for example *Lactobacillus acidophilus, Bifidobacterium animalis, Oenococcus oeni, Aspergillus niger, A. carbonarius, A. fumigatus, Saccharomyces cerevisiae,* and *Kloeckera apiculata* are able to detoxify mycotoxins [19–25].

Recent investigations demonstrate that *Saccharomyces cerevisiae* strains are able to remove OTA from plant raw material during fermentation of wine, beer, or sourdough [23, 26–29]. Some authors reported decreasing ochratoxin levels during winemaking [26, 27, 30–32]. In previous work, we suggested that physical binding to the yeasts biomass might be responsible for ochratoxin A decrease [33]. The compounds of the yeast's cell wall that are involved in the binding process are probably β-D-glucan and its esterified form. The oenological function of parietal yeast mannoproteins is reviewed by Caridi [34].

In the presented study, two wine strains of *Saccharomyces* yeasts were examined for their ability to remove ochratoxin A from grape and blackcurrant juice. The influence of OTA on fermentation and yeast metabolism were investigated too.

Materials and methods

Yeast strains

The following strains of *Saccharomyces cerevisiae* were used in this study: wine strains Malaga LOCK 0173, Syrena ŁOCK 0201, and bakery BS strain. These strains were selected for their ability of OTA removal in previous works [33, 35]. All strains were received from the Collection of Industrial Microorganisms of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz.

The yeast strains were stored on YPG slants (yeast extract 1 %, peptone 2 %, glucose 2 %, agar 1.5 %) at 4 °C and transferred to new slants once a month. Before carrying out the experiments, the strains were activated in 50 mL of liquid YPG medium in 100-mL flasks at 30 °C for 24 h on an orbital shaker (160 rpm). Yeasts biomass was collected by centrifugation (1,500*g*, 10 min) and suspended in 0.85 % NaCl (normal saline) at cell concentration of about 10^8 cfu/mL.

Additionally, biomass of baker's yeasts BS strains was suspended in normal saline (100 mg/mL), thermally inactivated in autoclave (121 °C, 20 min) and used in the further investigation.

Chemicals

All chemicals and solvents used were purchased from SIGMA-Aldrich, St. Louis, MO, USA, and were of analytical grade. Water from a Millipore Milli-Q system was used for all solutions, dilution, and the mobile phase for HPLC. Sulphuric acid (95–98 %) was obtained from J.T. Baker B.V. (Deventer, Holland). Organic acids used as standards were purchased from Supelco (Bellefonte, PA).

Ochratoxin A (SIGMA-Aldrich, St. Louis, MO, USA) was stored as stock solution in absolute ethanol (HPLC grade) at −20 °C. The concentration of OTA was 200 μg/mL.

Ochratoxin A removal assay

The OTA removal capacity of yeast was tested in YPG liquid medium as well as in media prepared from commercial concentrates of white grape juice and blackcurrant juice (Sonda Inc., Poland). The media for wine fermentation were prepared according to [36] by diluting white grape and blackcurrant concentrates to 12 Brix^o (1 Brix^o means 1 weight percentage of reducing sugars). YPG medium was sterilized at 117 °C, 15 min. All media were contaminated with ochratoxin A stock solution to obtain the initial concentration of 1 μg/mL OTA. The media were then inoculated with the yeast biomass suspended in 0.85 % NaCl. The initial concentration of yeast was about 10^6 cells/mL. The experiments in YPG medium were conducted in static conditions at 30 °C for 24 h. The fermentation of grape and blackcurrant medium in volume 200 mL was conducted in 250-mL flasks with a fermentation lock (gas trap) at 30 °C. During 10 days of fermentation, the weight loss and CO_2 production were monitored. The media not inoculated with the yeasts and inoculated but not contaminated with ochratoxin A served as control samples.

The possibility of binding ochratoxin A by thermally inactivated cells was tested in 50 mL YPG medium, grape, and blackcurrant wine obtained by fermentation by Syrena LOCK 0201 strain. The media were artificially contaminated by OTA to concentration 1 μg/mL. The baker's strain BS biomass was added to media to achieve concentrations of 5 mg/mL. The samples were kept at 30 °C for 24 h. All experiments were conducted in three independent samples.

Determination of cells number

A standard plate method on RBC medium (Rose Bengal Chloramphenicol, Merck, Darmstadt, Germany) was used for determination of the number of cells. The initial and final number of cells on musts and wines were estimated. In case of YPG medium, the number of cells before and during cultivation (at 2-h intervals) was determined. The results were expressed as cfu/mL.

The cell numbers on YPG medium were fitted to the Gompertz equation using an Excel add-in, DMFit 2.1 (Institute of Ford Research, Norwich, UK): $L(t) = A + C\exp\{-\exp[-B(t-M)]\}$.

The following growth parameters were estimated according to [37]:

$$\mu_{\max} = BC/e \quad (1)$$

where $\mu_{\max}$ is the maximum specific growth rate [h^{-1}], B and C are the constant from Gompertz equation

$$t\mathrm{Lag} = M - (1/B) \quad (2)$$

where tLag is the lag time (h), M and B are the constant from Gompertz equation.

Ochratoxin A analysis

The estimation of OTA in YPG medium and in raw materials after removing the biomass by centrifugation (1,500g, 10 min) was performed before (at time = 0) and after the fermentation or binding process with dead cells. Samples were purified by the use of immunoaffinity column OchraStar® (Romer Labs® Diagnostic GmbH, Tulln, Austria). Ochratoxin A was analysed by Finnigan™ Surveyor Plus™ HPLC System (Thermo Separation Products, Riviera Beach Fl., USA) equipped with an autosampler system, using a C18 Ace column (25 cm × 4.6 mm, 5 µm particles) at room temperature. Samples (100 µL) were injected (Rheodyne, Cotati, USA), eluted by water/acethonitrile/glacial acetic acid (99:99:2 v/v) at a flow rate of 1.0 mL/min, and detected with fluorescence detector (Finnigan Surveyor FL Plus Detector) at $\lambda_{\text{excitation}} =$ 330 nm and $\lambda_{\text{emission}} = 460$ nm.

The decrease of ochratoxin A in the medium in relation to the initial concentration was expressed in percentage.

Determination of fermentation products

The presence of fermentation products in wines (ethanol, glycerol, and acids) was determined according to Gutarowska and Czyzowska [38] by HPLC method using Finnigan Surveyor chromatograph equipped with a refractive index detector (Finnigan Surveyor-RI Plus detector), a diode array detector (Finnigan Surveyor-PDA Plus detector), and a Aminex HPX-87H column (300 × 7.8 mm). The chromatographic analysis was performed at 60 °C using sulphuric acid (5 mmol/L) as the eluent at a flow rate of 0.6 mL/min with a sample volume of 10 µL. Quantitation was based on the peak area measurement. This experiment was carried out only for one strain—Syrena LOCK 0201.

Statistical analysis

All experiments were conducted in triplicate. For the date statistical analysis, the Microcal ORIGIN ver. 6.0 software (Northampton, USA) was used. The resulting data were subjected to statistical analysis, including determination of the arithmetic mean, standard deviation, and variance analysis (one-way ANOVA test). Probability (P) values of < 0.05 were considered significant. For modelling of yeasts growth on YPG medium and estimating of growth parameters, the Gompertz model was used.

Results and discussion

There were no significant differences observed ($P < 0.05$) between ochratoxin A concentration before and after 10 days incubation of contamination media without yeasts (control samples). Both wine strains were able to decrease the initial OTA concentration in the artificial YPG medium (Table 1). A reduction of 21.0 and 35.4 % was measured for the strains Syrena LOCK 0201 and Malaga LOCK 0173, respectively. Similar results were reported by Bejaoui et al. A significant decrease of OTA levels in YPG medium (11–45 %) after 6 days of fermentation with oenological *Saccharomyces* strains was observed [27].

In the white grape must fermented with the two yeast strains, the OTA levels clearly decreased after fermentation, and reduction levels exceeding of 80 % were noted (Table 1). There are no significant differences ($P < 0.05$) between strains. The experiment with blackcurrant must

Table 1 The reduction of ochratoxin A in model and natural media

Strains	% Reduction of ochratoxin A (mean ± SD)		
	YPG + OTA[a]	GM + OTA	BM + OTA
Syrena LOCK 0201	21.0 ± 2.10[a]	85.1 ± 6.02[b]	65.2 ± 2.35[b]
Malaga LOCK 0173	35.4 ± 1.82[a]	82.8 ± 2.90[b]	10.7 ± 2.32[b]
BS (thermolized biomass)	54.1 ± 1.75[a]	64.4 ± 1.54[a]	62.4 ± 1.12[a]

YPG yeast extract, peptone, glucose medium, *GM* grape medium, *BM* blackcurrant medium, *OTA* ochratoxin A

Values represented the means of triplicate determinations ± SD, [a]Results after 24 h, [b]Results after 10 days of fermentation

Table 2 The effect of ochratoxin A on yeast's growth

Strains	Parameter	Medium					
		YPG + OTA	YPG	GM + OTA	GM	BM + OTA	BM
Malaga LOCK 0173	*t*Lag (h)	14.31 ± 0.46^{a}	10.63 ± 0.61^{b}	nd	nd	nd	nd
	μ_{max} (h^{-1})	0.33 ± 0.04^{a}	0.19 ± 0.01^{b}	nd	nd	nd	nd
	Biomass concentration ($\times 10^7$ cfu/mL)	5.03 ± 0.10^{a}	5.40 ± 0.10^{a}	1.16 ± 0.27^{b}	1.38 ± 0.27^{b}	0.57 ± 0.08^{c}	0.68 ± 0.13^{c}
Syrena LOCK 0201	*t*Lag (h)	16.35 ± 0.36^{a}	11.91 ± 0.26^{b}	nd	nd	nd	nd
	μ_{max} (h^{-1})	0.57 ± 0.01^{a}	0.367 ± 0.01^{b}	nd	nd	nd	nd
	Biomass concentration ($\times 10^7$ cfu/mL)	9.82 ± 0.46^{a}	15.37 ± 0.30^{d}	2.57 ± 0.17^{c}	2.74 ± 0.20^{c}	1.69 ± 0.52^{b}	2.03 ± 0.26^{b}

YPG yeast extract, peptone, glucose medium, *GM* grape medium, *BM* blackcurrant medium, *OTA* ochratoxin A, *nd* not determined

Values represented the means of triplicate determinations ± SD. Different letters in the same line indicate significant differences ($P < 0.05$)

shows differences between strains. Syrena LOCK 0201 strain is able to remove about 6 times more OTA than Malaga LOCK 0173 strain in the same condition. It demonstrated that removing of ochratoxin A from media depends on type of musts and on strains. These results showed the high capacity of both yeast strains to decreasing the OTA concentration during white wine processing and are in accordance with data published by Cecchini et al. [26]. They found that yeast strains after 36 days of white grape must fermentation remove about 50 % of the initial OTA amount (2 μg/L) and argued that the reduction of the mycotoxin was due to adsorption to the cell walls of the yeasts. Similar results were showed by Caridi et al. [30]. After 90 days of naturally OTA contaminated grape must fermentation, the amount of toxin decrease from 40 to 80 % depends on the strain. In both studies, the concentration of toxins was lower and the time of contact between the yeast and the OTA was longer than in our research.

The reduction of OTA level after treatment of contaminated GM and BM media after fermentation by thermally inactivated biomass of yeasts was observed too. From 54.1 to 64.4 % of initial ochratoxin A concentration was removed (Table 1). The higher adsorption from fermented GM and BM media than from YPG medium was observed. These results are corresponding with previous reports that described adsorption of some mycotoxins by heat-inactivated yeasts [27]. The authors found that the OTA removal from synthetic grape juice by heat-treated yeasts reached 75 % after 2 h of incubation, but the biomass concentration was higher than in our research. Better decontamination ability of killed cells against the viable cells indicates the physical nature of binding. The adsorption to cell wall is suggested as a mechanism of this phenomenon [27, 33]. Cell wall components play a significant role in mycotoxin binding, especially outer layer consisting of mannoproteins. Heating may cause changes in the surface properties of

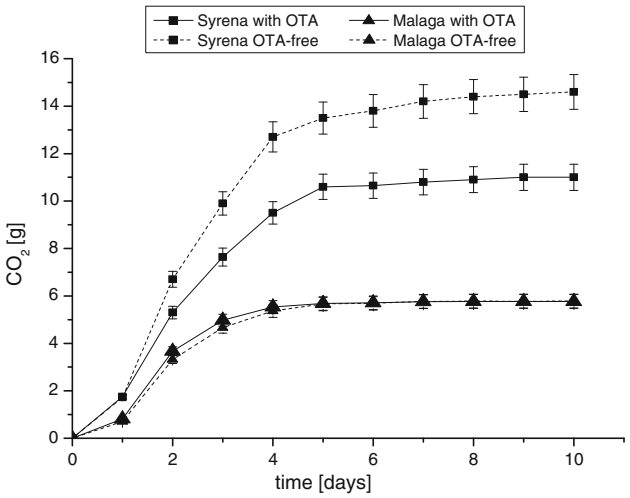

Fig. 1 Fermentation profiles in white grape medium

cells, for example denaturation of proteins or formation of Maillard reaction products. These products could possess more adsorption sites than viable cells. Moreover, the changes in cell wall thickness, increase of pore size, and permeability under heat treatment were observed [39]. The use of inactive dry yeasts in the oenological industry to improve technological processes, sensory characters, or to avoid negative impact of OTA on human health is recently reviewed by Pozo-Bayon et al. [40]. The application of killed cells as adsorbent for the purpose of elimination of ochratoxin A, for example from wine, is highly advantageous since such biomass does not change organoleptic features of end products. But the polyphenols may adsorb to the same binding sites on the surface of cells. It makes that this method is more useful rather for white wines than for red. It has been shown that various types of commercial fining agents using in oenology, such as activated carbon, silica gel, bentonite, and gelatin have the capacity to remove

OTA too [41]. According to Castellari et al. activated carbon and silica gel in the dosage of about 10 and 100 mg/L, respectively, are able to adsorb after 12 h about 50–60 % of OTA from red wines. The dosage of fining agents was about 50 times less than in our research, and the concentration of OTA was also low at 3.78 ng/mL. Activated carbon also removes anthocyanins and other polyphenols from wine, but only in high amount of adsorbent, exceeding 50 g/L [41].

In Table 2, the effect of OTA on yeast's growth is summarized. The inhibitory effect of OTA contamination on biomass yield was observed only for Syrena LOCK 0201 strain in YPG medium. The final cell number of this strain after 24 h in YPG medium with OTA was 9.8×10^7 cfu/mL, whereas in control sample, 1.5×10^8 cfu/mL. For Malaga LOCK 0173 strains on YPG medium, no differences were observed. The elongation of lag phase on contaminated YPG medium compared on toxin-free medium was noted (Table 2). In white grape and blackcurrant medium, the differences between the final cell number in the medium with OTA and the control were not statistically significant ($P < 0.05$). These results demonstrate the absence of a negative effect of a toxin on biomass yield. The effect of ochratoxin A on the fermentation rate measured as the weight loss (CO_2 evolution) during 10 days of fermentation was presented on Figs. 1 and 2. It was observed that for Syrena LOCK 0201 strain, the fermentation of white grape medium was more dynamic in the toxin-free medium than in the contaminated. In case of Malaga LOCK 0173 strain, the inhibitory effect of OTA on the fermentation kinetic was not found. The fermentation of blackcurrant medium was less effective. About 4.5 g of carbon dioxide was released, while in grape medium, it was 15 and 6 g for Syrena LOCK 0201 and Malaga LOCK 0173 strains, respectively. The inhibitory effect of ochratoxin A on the course of fermentation was observed (Fig. 2) in case of both strains.

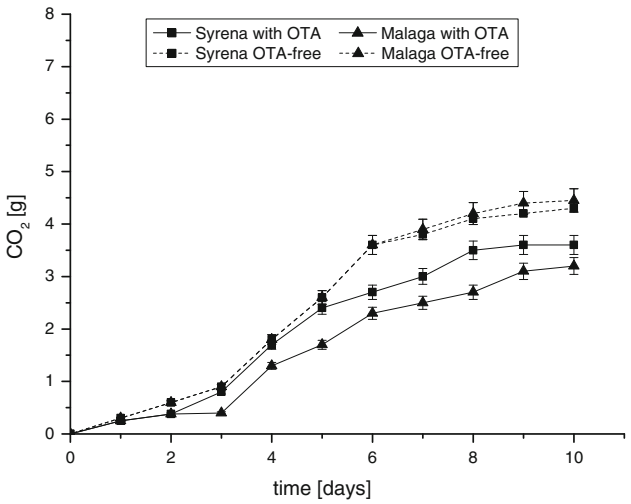

Fig. 2 Fermentation profiles in blackcurrant medium

Syrena LOCK 0201 strain was able to produce (in average) 73 and 49 g/L of ethanol on grape and blackcurrant medium, respectively, after 10 days. On BM, the production of glycerol was twice higher than on GM. The blackcurrant wines contented more acids: acetic, succinic, and citric than white grape medium (Table 3). There are no significant differences in amount of ethanol, glycerol, and acids ($P < 0.05$) between the media contaminated with ochratoxin A and toxin-free. The results of this part of the experiment indicated that contamination of raw material with ochratoxin A did not significantly influence the rate of fermentation and technological properties of winemaking. This statement is contrary to Bizaj et al. [42] which observed that the presence of OTA in synthetic media influenced yeasts metabolism, especially higher volatile acidity production. On the other side, according to Meca et al. [32], ochratoxin presence in must did not affect the alcoholic fermentation of Italian red wine Moscato.

Table 3 The influence of ochratoxin A on ethanol, glycerol, and acids content in wine fermented by Syrena LOCK 0201 strain

Product (g/L)	Medium			
	GM	GM + OTA	BM	BM + OTA
Ethanol	73.94 ± 1.69^a	72.23 ± 1.19^a	50.09 ± 1.06^b	48.13 ± 1.17^b
Glycerol	4.66 ± 0.28^a	4.54 ± 0.25^a	8.35 ± 0.37^b	8.16 ± 0.23^b
Acetic acid	0.49 ± 0.09^a	0.42 ± 0.08^a	1.91 ± 0.11^b	1.74 ± 0.12^b
Lactic acid	nd	0.13 ± 0.05	nd	nd
Succinic acid	0.31 ± 0.09^a	0.42 ± 0.07^a	nd	0.54 ± 0.06
Citric acid	0.46 ± 0.05^a	1.94 ± 0.06^c	10.11 ± 1.3^b	11.48 ± 1.65^b

GM grape medium, *BM* blackcurrant medium, *nd* not detected [under limit of detection (0.01 g/L)]

Values represented the means of triplicate determinations ± SD. Different letters in the same line indicate significant differences ($P < 0.05$)

Conclusions

Ochratoxin A contamination of grapes and grape products is a serious problem in oenological practise. The presented results showed that the application of selected strains of yeasts in biotechnological processes such as winemaking involving raw material contaminated with OTA might reduce the toxin contamination as well as the health risk related to human exposure to this toxin. Moreover, the application of heat-inactivated yeast's biomass for toxin adsorption gives new possibilities in oenology. Further studies are needed to clarify the mechanism of binding, as well as perform the economic calculation of cost-effectiveness of this method of wine decontamination.

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