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PhD RESEARCH IN "FOOD BIOTECHNOLGY" (cycle XXVI)

Selection and characterization of autochthonous *Saccharomyces cerevisiae* as functional starters for the removal of ochratoxin A in wine

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This PhD thesis is dedicated to my wife Cristina and my sons Francesca, Simone and Andrea
who have all made incredible and selfless sacrifices for me over many years that
I might someday have this privilege

«And the rain came down and there was a rush of waters
and the winds were driving against that house,
but it was not moved;
because it was based on the rock»

(Matthew 7:25)

STATEMENT OF ORIGINALITY

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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January 7, 2014

ABSTRACT

Scientific background: (i) Several studies have showed the key role of autochthonous yeasts in wine fermentation; (ii) Functional starter cultures represent a new frontier goal for the design of food products, as they show both the benefits of traditional starters and a health- or product-focused function; (iii) A particular kind of functional trait for wine yeasts could be the removal of mycotoxin ochratoxin A (OTA) as it is considered the principal safety hazard in the winemaking process; (iv) A variety of physical and chemical approaches to counteract the OTA problem were proposed; however, some limitations including the loss of nutritional and organoleptic qualities, the high cost and some practical difficulties were reported; (v) The most recent approach for the bioremediation of OTA in wine is the use of *Saccharomyces* and non-*Saccharomyces* yeasts able to bind the toxin to cell wall structures; (vi) OTA binding could be the result of weak hydrogen bonding, ionic or hydrophobic interactions.

Open questions: (i) Few data are available on the oenological traits of OTA-removing yeasts; (ii) Few data are available on the effect of temperature on the removal of OTA by yeasts and nobody studied in the past the role of sugar and nitrogen on this phenomenon; (iii) No data are available on the stability of OTA-yeast complex under winemaking conditions.

Aims: Selection and characterization of autochthonous *S. cerevisiae* as functional starters able to remove OTA, thus assuring wine quality and safety.

Planning of the research In the first part, 35 *S. cerevisiae* strains from 152 isolates from Uva di Troia cv., a red wine grape variety grown in the Apulian region (Southern Italy), were: (i) identified by PCR-RFLP analysis of the ITS region and DNA sequencing; (ii) differentiated at strain level by the amplification of interdelta region; (iii) studied for their phenotypical traits; (iv) analyzed for their technological performances and OTA-removal ability under *in vitro* conditions by a small-scale fermentation assay performed at different temperatures (25 and 30°C) and sugar levels (200 and 250 g l^{-1}) and compared to 4 commercial *S. cerevisiae* strains. In the second part, 3 selected strains were studied for their fermentative performances and OTA-removing ability under *in vitro* conditions by modulating time (0, 1, 3, 6 and 10 days), temperature (25 and 30°C), sugar (200 and 250 g l^{-1}) and diammonium phosphate (DAP) (300 mg l^{-1}). In the last part, the selected strains were characterized under *in vivo* conditions to investigate the ability to remove OTA during the fermentation of Uva di Troia grape must and assess the role of temperature (25 and 30°C), sugar (200 and 250 g l^{-1}) and addition of DAP (300 mg l^{-1}) on this phenomenon; the stability of OTA-yeast complex was studied by evaluating the amount of toxin released back after some washing treatments with phosphate-buffered saline (PBS, pH 3.5). Finally, the influence of OTA on the fermentation kinetic was investigated.

Materials and Methods: Phenotypical traits were assessed on the opportune laboratory media. Fermentation products were evaluated by Fourier Transform Infrared Spectroscopy (FTIR). OTA content was determined by enzyme linked immuno-sorbent assay (ELISA). For *in vivo* and OTA-release assays, ELISA coupled with immunoaffinity columns (IACs) was used.

Results: The most promising strain (*S. cerevisiae* W13) showed the ability to remove OTA under *in vitro* (6.00-57.21%) and *in vivo* conditions (30.69-53.79%), fermenting completely sugars and producing high ethanol and glycerol content, low volatile acidity amount, as well as, showing a high tolerance to single and combined fermentative stress conditions, β-D-glucosidase, pectolytic and xylanase activities, a low level of hydrogen sulphide production, a low-to-medium parietal interaction with phenolic compounds and no potential biogenic amines formation. In addition, *S. cerevisiae* W13 showed the lowest release of OTA after its biological removal during the alcoholic fermentation.

Significance and Impact of PhD research: For the first time, the ability of *S. cerevisiae* to remove OTA during alcoholic fermentation was used as an additional functional trait in a yeast-

characterization programme. Despite that native Uva di Troia cv. is commonly used as base wine for *controlled denomination of origin* (DOC) products in commercial wineries in the Apulian region, no studies have been carried out to improve its enological characteristics through the use of indigenous strains of *S. cerevisiae*. The present PhD thesis is the first report concerning the enological and biotechnological potential of autochthonous *S. cerevisiae* strains isolated from Uva di Troia grape. Moreover, this PhD project is the first attempt to investigate the stability of OTA-yeast complex under winemaking conditions; the release of the toxin was strictly strain-dependent. Finally, the novelty of this PhD thesis relies also upon the effect of the most important factors of wine fermentation (temperature, sugar and nitrogen concentration, nature of the yeast strain) on the removal of OTA; the toxin removal was affected by the kind of strains and/or by the nutritional and environmental conditions.

Future trends: (i) A future perspective could be a focus on the role of cell wall charge on the complex phenomena of OTA removal and release to minimize the amount of the toxin released back into the medium by the starter; (ii) The phenomena should be investigated in terms of the genetic determinants of cell wall properties in *S. cerevisiae* to help the design of strategies for the genetic improvement of potential functional starters; (iii) *S. cerevisiae* W13 should be used in a pilot-fermentation to assess its ability to compete with the natural microbiota of grape must.

Key words: wine; *Saccharomyces cerevisiae*; yeast selection; functional starter; ochratoxin A removal; ochratoxin A release.

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PUBLICATIONS ARISING FROM THIS PhD PROJECT

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Chapter 1. INTRODUCTION

1.1. SCIENTIFIC BACKGROUND

Safety and security have generally remained among the basic human needs in today's changing world. Over the last years, ensuring the safety of food has been a major focus in international and national acts. Both microbiological and chemical hazards are of concern. Among chemical hazards, the contamination of food and feed by mycotoxins [toxic metabolites of fungi] have been recently characterized by the World Health Organization (WHO) as significant sources of food-borne illnesses (Anly and Bayram 2009).

The Food and Agriculture Organization (FAO) estimates that 25% of the world's agricultural commodities are contaminated with mycotoxins, leading to significant economic losses. Up to now, approximately 400 secondary metabolites with toxigenic potential produced by more than 100 moulds have been reported (Jard et al. 2011). Increasing interest has been receiving ochratoxin A (OTA) due to its toxic effects and high incidence in a wide range of food products and beverages, including wine (Pozo-Bayón et al. 2012).

OTA is a nephrotoxin that exhibits immunosuppressive, teratogenic and carcinogenic properties. The International Agency for Research on Cancer (IARC) classified in 1993 OTA as a possible human carcinogen (group 2 B). Several nephropathies affecting animals as well as humans have been attributed to OTA. In humans, OTA is frequently reported as the possible causative agent of endemic kidney disease observed in the Balkans (Balkan Endemic Nephropathy and related Urinary Tract Tumours) (Quintela et al. 2013).

OTA was discovered in the mid-1960s and was for the first time found as a natural contaminant in cereals at the end of the 1960s. Since 1996 the presence of OTA has been found in wine as a consequence of fungal growth on grapes (Jørgensen 2005). Although *Penicillium verrucosum* and *Aspergillus ochraceus* are considered to be the main OTA producing species, there is a strong evidence for the role of *Aspergillus carbonarius* in OTA contamination in wine grapes (Quintela et al. 2013). *A. carbonarius* exists as a saprophyte in the top layer of soil beneath vines and grows in berries injured by biotic

agents, pests and diseases, and also by abiotic factors. Because of its active role in transporting spores into injured berries, the grape berry moth (*Lobesia botrana*) is a major cause of colonization by black *Aspergillus* species (section *Nigri*) (Cubaiu 2008). The temperature, the moisture, the aeration, the period of infection and the interaction between different fungi play a significant role in mycotoxin diffusion (Esti et al. 2012).

The level of OTA varies considerably among various types of wines and vine products, regions and vintages (Solfrizzo et al. 2010). Generally red wines have higher levels than white wines, due to the increased time of contact between berry skins and grape juice during the mashing stage. According to the SCOOP (Scientific Cooperation on Questions relating to Food) task 3.2.7 report of European Commission, after performing a survey on 1470 wine samples, the OTA mean level was 0.36 μg l^{-1}; however, a level of 15.6 μg l^{-1} was reported in red wine from Southern Europe (Cubaiu 2008). Several surveys carried out in different European Countries as well as Morocco, Japan, and Australia have confirmed the frequent presence of OTA on grape products and wine at levels ranging from 0.01 to 3.4 μg l^{-1} (Battilani et al. 2003).

Based on the available scientific toxicological and exposure data, the European Commission in 2005 set the limit of OTA for wine, grape juice, grape nectar and grape must intended for direct human consumption in 2 μg l^{-1} by the Regulation (EC) N°123/2005 that have been updated with the Regulation (EC) N°1881/2006 (Esti et al. 2012). However, many trade agreements require lower limits than those adopted by the Regulation and some of these agreements require OTA <0.5 μg l^{-1} (Solfrizzo et al. 2010).

Both preventive and corrective approaches have been used to reduce the incidence of OTA contamination; the prevention strategies include the use of biocontrol agents and fungicides against *A. carbonarius*, insecticides against *Lobesia botrana*. However these approaches cannot completely prevent the OTA problem, and severe contamination of wine can occur especially for some susceptible grape varieties in certain high risk regions or vintages with climatic conditions promoting the infection by *A. carbonarius* (Solfrizzo et al. 2010). On the other hand, some physical methods to reduce OTA level in wine have been proposed. Physical decontamination of OTA involves first the removing of mouldy grapes or bunches before entering in the winemaking process (Leong et al. 2006). According to Quintela et al. (2013) this method may be able to reduce OTA incidence up to 98%; but it might not be economically feasible for the wine industry. Solfrizzo et al. (2010) found that OTA can be effectively removed up to 50-65% by

repassage of contaminated musts or wines over no or little contaminated pomaces from the same or different grape varieties. The wine quality parameters were only affected when the pomace came from a different variety, thus the effect was related to the intrinsic characteristics of the pomace variety.

Wine filtration through a 0.45 μm membrane reduced OTA level by 80%; on the other hand, a filtration through a 10 μm membrane does not reduce significantly the level of the toxin. Thermal treatments do not affect the OTA concentration (Quintela et al. 2013).

Another approach to remove OTA from contaminated wine involves the use of chemical materials with the capacity to adsorb and immobilize the mycotoxin. Activated carbon, bentonite, egg albumin, $AlCl_3$ activated gelatin, microcrystalline potassium caseinate, PVPP (polyvinylpolypyrrolidone) have been reported to be able to remove OTA in wine (Quintela et al. 2013); however, this approach could decrease wine quality by removing color, aroma, flavor, or other desirable wine characteristics (Fernandes et al. 2007). In addition, results in the OTA removal in wine relied upon OTA contamination, the fining agent used, as well as its type and dosage. Finally, some fining agents commonly used in winemaking process could cause adverse reactions in susceptible wine consumers (Quintela et al. 2013).

Oak wood fragments can be used to reduce the levels of OTA in wine. The effectiveness of the treatment depended on the wood format (chips or powder), quantity, time of contact and wine composition. The detailed rules for the use of such treatment were established in 2006 by the Commission Regulation (EC) N°1507/2006; however, its use is forbidden in wines produced in some European regions (Quintela et al. 2013).

Since all mycotoxins are very stable substances, and no physical or chemical treatment can be applied without altering the nutritional value of the grain or causing a high rise in costs (Jard et al. 2011), biological decontamination of mycotoxins using microorganisms or microbial-derived enzymes is the most recent approach to control OTA incidence (Quintela et al. 2013). Enzymes with carboxypeptidase A activity (CPA) can hydrolyze the amide bond in OTA molecule with the production of L-*β*-phenylalanine and ochratoxin α, which is less toxic than OTA (Rodriguez et al. 2011). The use of atoxigenic *Aspergillus niger* strains as carboxypeptidase sources has been suggested; in addition, some strains of *A. niger* strains could produce lipases, playing an active role in OTA decontamination. A carboxypeptidase present in *Phaffia rhodozyma* can also degrade OTA

up to 90%. Likewise, degradation of OTA by some strains of *Streptococcus*, *Bifidobacterium*, *Lactobacillus*, *Butyribrio*, *Phenylobacterium*, *Pleurotus*, *Bacillus* and *Acinetobacter* and by *Aspergillus* (*A. fumigatus*, *A. niger*, *A. carbonarius*, *A. japonicus*, *A. versicolor*, *A. wentii* and *A. ochraceus*), *Alternaria*, *Botrytis*, *Cladosporium*, *Phaffia*, *Penicillum* and *Rhizopus* (*R. stolonifer* and *R. oryzae*) have been reported. In particular, these genera have been shown to enzymatically degrade OTA *in vitro* up by more than 95%; moreover, some of them have shown detoxifying properties in *in vivo* assays (Amézqueta et al. 2009). However, there are some questions upon the toxicity of products of enzymatic degradation and the undesired effects of non-native microorganisms on food quality (Cubaiu 2008).

Microorganisms are also able to remove OTA by adsorption onto the cell surface components. Biosorption is generally based on a set of chemical and physical mechanisms leading to the immobilisation of a solute component on the microbial cell wall components (Ringot et al. 2007). For example, adsorption effects were reported by Turbic et al. (2002), who found that heat and acid treated cells from two strains of *Lactobacillus rhamnosus* showed a higher removal yield than viable cells. The strains removed from 36 to 76% in a phosphate solution (pH 7.4) after 2 h at 37°C. Similarly, Piotrowska and Zakowska (2005) found the adsorption of OTA onto some *Lactobacillus* and *Lactococcus* strains, being *Lb. acidophilus* CH-5, *Lb. rhamnosus* GG, *Lb. plantarum* BS, *Lb. brevis*, and *Lb. sanfranciscensis* the best strains with removing yields of *ca.* 50%.

Also yeasts are able to adsorb OTA. According to Ringot et al. (2007), yeast biomass may be regarded as a good source of adsorbent material, due to the presence in the cell wall of some specific macromolecules, such as mannoproteins and β-D-glucans. For example, the cell wall of *Saccharomyces* is constituted by 25-50% of mannoproteins and their degree of glycosilation is variable. Mannoproteins are partially water-soluble components and are released throughout and mainly at the end of alcoholic fermentation. At wine pH mannoproteins carry negative charges, so they may establish both polar and non-polar interactions with OTA. In several yeasts the glycan portion of mannoproteins is variable and this may explain the differences in OTA removal ability exerted by different strains (Quintela et al. 2013). Other compounds, like phenols, proteins, organic acid, and colloidal particles showed the same ability of OTA to react with different compound of the cell wall, thus they could be considered as competitors in the OTA detoxification process (Meca et al. 2010). Therefore, Piotrowska et al. (2013) reported that bioremediation by yeasts could be more useful in white wines rather than

in red. On the contrary, according to Cecchini et al. (2006) it is probable that mannoproteins interact with polyphenol compounds of red must and could raise the ability to adsorb OTA.

Despite the strong evidence of OTA adsorption to cell walls (Bejaoui et al. 2004; Garcia-Moruno et al. 2005; Ringot et al. 2007; Silva et al. 2007; Nunez et al. 2008; Piotrowska 2012), Angioni et al. (2007) found that some yeasts could reduce OTA levels in wine, although they did not adsorb OTA. Thus, these authors suggested the existence of a possible pathway for the degradation of the toxin, different from the mechanism involving the L-*β*-phenylalanine and ochratoxin α; however, they could not find any product of hydrolysis.

Piotrowska (2012) reported that cells without cell wall (protoplast) lost the ability to adsorb OTA. Bejaoui et al. (2004) verified that heat and acid treated cells could bind significantly higher levels of OTA than the viable ones. Viable yeasts bound up to 35% and 45% of the OTA, depending on the medium and strain, while heat and acid treated cells bound a maximum of 75%. On the other hand, Nunez et al. (2008) tested whole yeast cells and yeast cell walls to remove OTA in a wine model system; heat treatment (85°C for 10 min) increased the OTA removal capacity from 0.8 and 18.8 to 95.4 and 95%, respectively. A possible explanation was suggested by Piotrowska et al. (2013) who reported that heating may cause changes in the surface properties of cells, like the denaturation of proteins or the formation of Maillard reaction products. These products could harbour higher adsorption sites than viable cells with an enhancement of OTA removal.

Different yeasts by-products, yeast walls and hulls and inactivated yeasts were tested by Silva et al. (2007) and the maximum reduction of the toxin was generally achieved after 8 days of contact at 20°C; the addition of 100 g hl^{-1} of yeast walls, 40 g hl^{-1} of yeast hulls or 200 g hl^{-1} of active dry yeasts decreased the content of OTA by 40-50%. Ringot et al. (2007) reported the adsorption of OTA onto yeast industry by-products: a vinasse containing yeast cell walls, a purified yeast β-glucan, and a yeast cell wall fraction. The cell wall fraction was able to bind 95-100% of OTA. Garcia-Moruno et al. (2005) showed that the percentages of OTA reduction were different using white and red lees-wine; after 90 min of contact (20 g l^{-1} of lees), the white lees-wine reduced OTA by 50.4% and 63% while the red lees-wine decreased the toxin by 22.8% and 31.7%; after 7 days the OTA reduction was *ca.* 70% with the white lees and *ca.* 50% with the red lees.

1.2. OPEN QUESTIONS

Some authors identified yeasts from *Saccharomyces* and non-*Saccharomyces* genera able to decrease OTA content throughout wine fermentation stage between 30-100% (**Table 1**). However, a drawback in the literature is that few data are available on the oenological traits of OTA-removing yeasts, whilst this aspect is of great concern for the possible selection of strains acting at the same time as starters and as biological tools to remove the toxin.

In recent years, functional starter cultures represent a new frontier goal for the design of food products, as they show both the benefits of traditional starters and a health- or product-focused function (Bevilacqua et al. 2012). In this context, a particular kind of functional trait for wine yeasts could be the removal of OTA to complement the classical oenological characterization based on technological and qualitative key-traits, such as the tolerance and high ethanol production, the exhaustion of sugars, the growth at high sugar concentration, a good glycerol production, the growth at high temperatures, a low hydrogen sulphide and volatile acidity production, the resistance to sulphur dioxide, and a good enzymatic profile (Nikolaou et al. 2006).

On the other hand, although many papers focused on the role of some physico-chemical factors (temperature, sugar and nitrogen) on the course of wine fermentation (Bely et al. 2003,2008; Torija et al. 2003; Hernández-Orte et al. 2006), few data are available on the influence of temperature on removal of OTA by yeasts (Patharajan et al. 2011) and, to the best of our knowledge, nobody studied in the past the role of sugar and nitrogen on this phenomenon. In fact, some oenological parameters can modify the surface properties of cell wall (Vasserot et al. 1997), thus they could affect the binding capacity of yeasts.

Finally, when evaluating a microorganism as a potential decontaminating agent, the assessment of the stability of toxin-cell complex could be a key trait, as toxin release would have negative health implications (Peltonen et al. 2001). A drawback in the literature is that no data are available on the stability of OTA-yeast complex under winemaking conditions, although this issue is very important due to the fact that OTA binding to yeast cell-wall could be the result of weak hydrogen bonding, ionic or hydrophobic interactions (Cecchini et al. 2006). In fact, the strains removing efficiently the toxin could release it back; thus, the selection of the starter should take into account both the removal and the binding ability of OTA.

1.3. AIMS AND PLANNING OF THE RESEARCH

My PhD thesis was focused on the selection and characterization of autochthonous *S. cerevisiae* as functional starters able to remove OTA, thus assuring wine quality and safety.

My PhD project involved 3 different steps. In the first part, 35 *S. cerevisiae* strains from 152 isolates from Uva di Troia cv., a red wine grape variety grown in the Apulian region (Southern Italy), were: (i) identified by PCR-RFLP analysis of the ITS region and DNA sequencing; (ii) differentiated at strain level by the amplification of interdelta region; (iii) studied for their phenotypical traits, including the tolerance to fermentative stress conditions, the enzymatic potential, parietal interaction with phenolic compounds, hydrogen sulphide production and amino acid decarboxylation; (iv) analyzed for their technological performances and OTA-removal ability under *in vitro* conditions by a small-scale fermentation assay performed at different temperatures (25 and 30°C) and sugar levels (200 and 250 g l^{-1}) and compared to 4 commercial *S. cerevisiae* strains.

The main goal of the second part of PhD thesis was to study 3 selected strains of *S. cerevisiae* for their fermentative performances and OTA-removing ability under *in vitro* conditions by modulating time (0, 1, 3, 6 and 10 days), temperature (25 and 30°C), sugar (200 and 250 g l^{-1}) and diammonium phosphate (DAP) (300 mg l^{-1}).

In the last part of this PhD, the selected strains of *S. cerevisiae* were characterized under *in vivo* conditions to investigate the ability to remove OTA during the fermentation of Uva di Troia red must and assess the role of temperature (25 and 30°C), sugar (200 and 250 g l^{-1}) and addition of DAP (300 mg l^{-1}) on this phenomenon; the stability of OTA-yeast complex was studied by evaluating the amount of toxin released back after some washing treatments with phosphate-buffered saline (PBS, pH 3.5). Finally, the influence of OTA on the fermentation kinetic was investigated.

Chapter 2. MATERIALS AND METHODS

2.1. AUTOCHTHONOUS YEASTS ISOLATION AND REFERENCE STRAINS

The sampling was carried out in a vineyard of Uva di Troia red wine variety (*Vitis vinifera* L.) located in the Apulian region (Foggia, Southern Italy). Healthy and undamaged grapes (from 8 vines, 1000 g of grapes *per* vine), immediately after harvesting (September, 2008), were collected in sterile bags; serial dilutions of grape samples, previously homogenized in sterile Ringer solution (Oxoid, Milan, Italy), were plated in duplicate on Wallerstein Laboratory (WL) agar (Oxoid), which allows to discriminate yeast species by colony morphology and colour (Pallmann et al. 2001). A total of 152 isolates, exhibiting *Saccharomyces* morphology on WL agar and randomly selected from plates, were purified by repeated streaking on the same medium. Thirty-five isolates were further used in this research.

Three commercial strains (EC1118, RC212 and BM45 from Lallemand Inc., Montreal, Canada) and a collection isolate of *S. cerevisiae* (DBVPG6500 from Industrial Yeasts Collection of the University of Perugia, Italy) were used as references in inoculated fermentations.

2.2. MOLECULAR IDENTIFICATION

The isolates were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacers (ITS), as described in Esteve-Zarzoso et al. (1999); 5.8S-ITS PCR fragments, generated by amplification with ITS1/ITS4 primers (White et al. 1990), were sequenced by Primm Biotech (Milan, Italy) to confirm the genotypic identification. The sequences were compared with those available in the GenBank database using the Basic Local Alignment Search Tool version 2.2.27 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. GENOTYPIC CHARACTERIZATION

The isolates were characterized by interdelta analysis, using PCR primers described by Legras and Karst (2003), i.e. delta12: 5'-TCAACAATGGAATCCCAAC-3' and delta21: 5'-CATCTTAACACCGTATATGA, following the procedure described by Capece et al.

(2012). PCR was performed with Taq PCR Core kit (Qiagen, Santa Clarita, CA, USA). Electrophoresis gels were scanned with Versadoc system (Bio-Rad, Richmond, CA, USA), converted to TIFF images, compared and analyzed with FPQuestTM software (BioRad Laboratories, Richmond, USA); then, the electrophoretic patterns were grouped, and analyzed for the similarity through the Dice coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA).

2.4. ANALYSIS OF YEAST TOLERANCE TO SEVERAL STRESS CONDITIONS

The yeasts were studied in relation to their growth on ethanol (6-24%, vol/vol), sulphur dioxide (150-1000 ppm), glucose (200, 250 and 300 g l^{-1}), fructose (200, 250 and 300 g l^{-1}), sucrose (200, 250 and 300 g l^{-1}), copper (100-1000 ppm), acetic acid (3.5 g l^{-1}), temperature (37 and 42°C); moreover, some combined stress were evaluated, i.e. 10% ethanol + 150 ppm sulphur dioxide, 250 g l^{-1} sucrose + incubation at 37°C, 10% ethanol + incubation at 37°C. YPD agar (yeast extract 10 g l^{-1}; bacteriological peptone 20 g l^{-1}; glucose 20 g l^{-1}; 12 g l^{-1} agar technical no. 3), buffered to pH 3.5 and supplemented with the adequate amounts of ethanol, sulphur dioxide, glucose, fructose, sucrose, copper and acetic acid, was inoculated with the target strains and incubated at 30°C or at 37-42°C (temperature stress) for 1-5 days. For each test a positive control was used, i.e. YPD agar, buffered to pH 3.5, inoculated with yeasts and incubated at 30°C. The experiments were performed in duplicate over two different batches.

2.5. ENZYMATIC ACTIVITIES

The following enzymatic activities were assessed: β-D-glucosidase on arbutin (Comitini et al. 2011), cellobiose (Daenen et al. 2008), esculin (Pérez et al. 2011) and 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) (Fiore et al. 2005); pectolytic and xylanolytic activities (Bevilacqua et al. 2009); protease (Strauss et al. 2001); esterhydrolase and glycosidase (Comitini et al. 2011); β-D-xylosidase (Fiore et al. 2005). β-D-glucosidase on esculin, pectolytic and xylanolytic activities were also studied at wine pH and wine ethanol concentration. Three different combinations were tested: a) 10% of ethanol; b) pH 3.5; c) pH 3.5 and 10% ethanol. The enzymatic activities were done in duplicate; non inoculated plates were used as negative controls.

2.6. OTHER OENOLOGICAL CHARACTERISTICS

The ability to produce hydrogen sulfide was tested by using a qualitative method based on colony appearance on Biggy agar (Orlić et al. 2010). Decarboxylase activity was determined using the method of Nikolaou et al. (2006); histidine, tyrosine, phenylalanine, tryptophane, lysine, leucine, ornithine and arginine were used as precursor amino acids. The assay was performed under anaerobic conditions using an anaerobic jar system form AnaeroGen (Oxoid). Interaction with phenolic compounds was determined using the methodology of Caridi et al. (2002). Un-inoculated plates were used as negative controls; the tests were performed in duplicate.

2.7. INOCULATED FERMENTATIONS UNDER *IN VITRO* AND *IN VIVO* CONDITIONS AND OTA REMOVAL

The fermentation under *in vitro* conditions (1st and 2nd part of PhD thesis) was assessed in the synthetic medium described by Lopes et al. (2007), with some modifications. This medium contained: 100 g l^{-1} glucose (C. Erba, Milan, Italy), 100 g l^{-1} fructose (Sigma-Aldrich, Milan, Italy), 10 g l^{-1} yeast extract (Oxoid), 1 g l^{-1} ammonium sulphate (J.T. Baker, Milan, Italy), 1 g l^{-1} potassium phosphate (C. Erba), 1 g l^{-1} magnesium sulphate (J.T. Baker); a second test was run using 250 g l^{-1} of reduced sugars by addition of glucose:fructose (1:1). Yeast assimilable nitrogen (YAN) was 250 mg l^{-1}; when diammonium phosphate (DAP) (Sigma-Aldrich) was added, the YAN content increased to 310 mg l^{-1}. The medium was sterilized at 121°C for 15 min. After the sterilization, the pH of the medium was decreased to 3.5 through a solution of citric acid (10 g l^{-1}) (Sigma-Aldrich).

Uva di Troia grape must (harvested 2011) was used to test the technological performances under *in vivo* conditions (3rd part of PhD thesis). The final reducing sugar concentration was adjusted to 200 or 250 g l^{-1} by addition of glucose:fructose (1:1). Two different experiments were performed with and without DAP addition (300 mg l^{-1}); the two samples had 174 and 114 mg l^{-1} YAN, respectively. The must was steam-sterilized at 90°C for 15 min (Ciani and Maccarelli 1998). Finally, tartaric acid (85%, wt/vol) (Sigma-Aldrich) was aseptically added to adjuste the pH to 3.5 (Du et al. 2012).

Flasks (150 ml in size) containing 100 ml of the media (the synthetic one and grape must) were used for fermentation trials; the media were inoculated with yeasts to 6 log cfu ml^{-1} from YPD medium overnight cultures (13 strains selected from the 35 isolates

as well as the reference strains of *S. cerevisiae* in 1st part, the strains W13, W28 and W46 in 2nd and 3rd steps), OTA added (2 μg l^{-1}) (Sigma-Aldrich) and the surface was covered with a thin layer of sterilised paraffin oil (10 ml *per* flask) in order to avoid air contact. A control sample was prepared with media containing OTA but without yeasts. In addition, a control with inoculated media not containing OTA was prepared for *in vivo* assay. The samples were incubated at 25 or 30°C without shaking. The weight loss of the fermenters was monitored and the fermentation considered finished when residual sugar concentration was <2 g l^{-1}; then, the flasks were shaken to thoroughly mix the contents and get all the cells and the products in suspension. Samples were collected at end of fermentation or at different time points according to the details reported in **Table 2**.
Three parameters were assessed on the fermented media: (1) OTA concentration and (2) the main fermentation products, after a centrifugation at 2100 *g* for 10 min at 4°C to remove yeast cells; (3) the viable cell count.

2.7.1. Evaluation of OTA release by yeasts after in vivo **fermentations**

For determining the possible release of OTA by yeasts after its biological removal under *in vivo* conditions, the method described by Serrano-Niño et al. (2013) was used, modified as follows: yeast pellet from the fermentation trial was suspended in 10 ml phosphate-buffered saline (PBS), mixed for 15 s and incubated at 37°C for 5 min; thereafter, yeast cells were centrifuged (2100 *g*, 10 min, 4°C) and the supernatant collected to assess the amount of OTA released back into the washing medium. This protocol was repeated up to 3 times. The PBS solution was prepared as reported by Castellari et al. (2000), using 0.2 g l^{-1} potassium chloride (J.T. Baker), 0.2 g l^{-1} potassium dihydrogenphosphate (C. Erba), 1.16 g l^{-1} anhydrous disodium hydrogenphosphate (C. Erba) and 8 g l^{-1} sodium chloride (Sigma Aldrich) in distilled water. The pH of PBS was adjusted to 3.5 with HCl 1 M (Topcu et al. 2010).

2.7. ANALYTICAL DETERMINATIONS AND YEAST VIABILITY

The quantitative analysis of OTA in the samples was based on enzyme linked immuno-sorbent assay (ELISA) method using Ridascreen®Ochratoxin A 30/15 ELISA kit (Art. No. R1311). For *in vivo* and OTA-release assays, ELISA coupled with immunoaffinity columns (IACs) were carried out using RIDA Ochratoxin A columns (Art. No. R1303). The test procedures were performed following the protocols provided by the

manufacturer (R-Biopharm; Darmstadt, Germany). The optical density of the reaction product was determined using ELISA 96-well plate reader (Bio-Rad Laboratories, Hercules, CA; Model No.680) set to 450 nm wavelength. The results were reported as percentages of OTA removed by yeasts from the medium. The experiments were performed in duplicate, over two different batches.

Residual sugars, ethanol, glycerol and volatile acidity were determined by Fourier Transform Infrared Spectroscopy (FTIR) by employing the WineScan FT120 instrument (FOSS Analytical, Hillerod, Denmark; software version 2.2.1) according to the supplier's instructions.

YAN content was evaluated through K-LARGE enzymatic kit (Megazyme, Bray, Ireland) according to the manufacturer's instructions.

For the yeast viability, a standard plate method on YPD agar was carried out. The plates were incubated at 30°C for 2-5 days.

2.8. STATISTICAL ANALYSIS

Statistical analyses were performed using the software STATISTICA for Windows (StatSoft, Inc., Tulsa, OK, USA; software version 10.0.1011.0).

Data obtained by phenotypical characterization were analyzed through Cluster Analysis, using the single linkage percent disagreement method; the results of the different assays were converted into qualitative codes, as reported in **Table 3**.

Data from small-scale fermentations (1st part of the thesis, see the planning of the research) were analyzed through one and two-way analysis of variance (ANOVA) and Tukey's test as post-hoc comparison test ($P<0.05$).

Data obtained by *in vitro* characterization of selected strains (2nd part of PhD thesis) were analyzed through one-way ANOVA and Tukey's test as the *post-hoc* comparison test ($P<0.05$). Afterwards, OTA reduction, cell level (log cfu ml^{-1}), ethanol, glycerol and volatile acidity contents, and the level of residual sugars were used as input values for a multiple regression approach (forward method); temperature (25 and 30°C), sugar (200 and 250 g l^{-1}), DAP content (0 and 300 mg l^{-1}) and time of sampling (immediately after the inoculation and after 1, 3, 6 and 10 days) were used as independent factors. The significance of the approach was evaluated through the standard error associated to the model, the adjusted regression coefficient and the Fisher test value ($P<0.05$).

Data obtained by *in vivo* characterization (3rd part of PhD thesis) (OTA removal, ethanol, glycerol and volatile acidity contents) were analyzed through one-way ANOVA and Tukey's test as the *post-hoc* comparison test ($P<0.05$). Therafter, the mean values of OTA removal, ethanol, glycerol and volatile acidity contents were used as the input data for a DoE analysis; temperature (25 and 30°C), sugar (200 and 250 g l^{-1}) and DAP content (0 and 300 mg l^{-1}) were used as independent variables or factors; each of them was set at two different levels ("-1" and "+1", respectively the minimum and the maximum levels of each variable). The analysis was performed thorugh the option DoE/$2^{(k-p)}$ standard designs/2 way interactions; OTA removal, ethanol, glycerol and volatile acidity contents after fermentation were used as dependent variables.

The software uses a multiple regression approach/intercept option to highlight the significant effect of each factor individually, as well as of their two way interaction (i.e. the interaction between two factors); the output is a table reporting for each factor or interaction the mathematical coefficient (i.e. the strength), the standard error of the coefficient, the 95%-confidence interval and the significance (t-test, evaluated as the ratio of the mathematical coefficient vs its standard error). The significance is used to build a table for the effect estimates, whilst the mathematical coefficients are used to predict the value of the dependent variable for many combinations of the design and draw the three-dimensional/contour plots.

Chapter 3. RESULTS AND DISCUSSION

3.1. SELECTION OF AUTOCHTHONOUS *Saccharomyces cerevisiae* STRAINS AS FUNCTIONAL WINE STARTERS

Despite the availability of several commercial yeasts to accomplish must fermentation, some oenologists admit that good results can be obtained only with autochthonous starters, isolated from the micro-area where wines are produced (Francesca et al. 2010). The use of local selected yeasts is believed to be much more effective as they are better adapted to the different wine-producing regions with their respective grape varietals, viticultural practices and winemaking techniques (Regodón et al. 1997).

A pressing necessity of Apulian region wine industry is to adopt innovative systems to guarantee and exalt the peculiar attributes of regional wines (Grieco et al. 2011). In this context, the characterization of autochthonous yeast strains is essential for wine-makers wishing to establish microbiological control of their winemaking process and to take full advantage of the potential of their regional territory for improving the production or making novel products (Capece et al. 2011). To date, several investigations have described the enological performances of *S. cerevisiae* strains isolated from red Apulian grapes such as Primitivo (Grieco et al. 2011), Negroamaro (Tristezza et al. 2012) and an ancient and recently rediscovered grape cultivar denoted as Susumaniello (Tristezza et al. 2013). No studies on the selection of native *S. cerevisiae* strains have been carried out to improve the enological characteristics of Uva di Troia cv., a native grape variety commonly used for the production of high quality red wines marked as *controlled denomination of origin* (DOC) (Genovese et al. 2013).

In the first part of PhD thesis, a polyphasic approach consisting in the genotypic characterization of 35 *S. cerevisiae* strains from 152 isolates from Uva di Troia grapes, the evaluation of the phenotypic traits, as well as the fermentative performances in a model system at 25 and 30°C and with different sugar amounts (200 and 250 g l^{-1}), was used as a suitable approach to select wine starters of *S. cerevisiae*; furthermore, the ability to remove OTA was used as an additional functional trait to improve the safety of wine.

3.1.1. Molecular identification and genotypic characterization of S. cerevisiae isolates

Some authors reported that the unambiguous taxonomic identification of *Saccharomyces* wine strains at the species level requires combinations of several methods (Csoma et al. 2010). Therefore, the genetic profiles generated by rDNA ITS-PCR-RFLP analysis were coupled with DNA automatic sequencing analysis of PCR products. All the isolates displayed a PCR pattern of 320, 230, 180 and 150 bp with *Hae*III, 365 and 155 bp with *Hinf*I, and 385 and 365 bp with *Cfo*I corresponding to those of *S. cerevisiae*. A 99-100% of identity according to BlastN search of ribosomal RNA gene sequences in NCBI database was found (data not shown). Molecular results were also confirmed by sequencing; the accession numbers were reported in **Figure 1**. Moreover, the amplification of interdelta region was used to highlight the degree of intraspecific diversity in *S. cerevisiae* population. This method is rapid, reproducible and very sensitive (Capece et al. 2012). As the main result, 13 different clusters (designed by the capital letters from I to XIII) were pointed out (**Figure 1**). Considering the ratio between the number of isolates and the number of patterns as an approximate index of the biodiversity, our results corresponding to a higher diversity than the one detected in some other viticulture regions (Schuller et al. 2005); however the present PhD thesis is not a survey on genetic diversity of autochthonous oenological yeasts, for which a large-scale study of the vineyard-associated *S. cerevisiae* strains was required (Valero et al. 2007).

The cluster VIII and XIII included the higher number of strains (5 strains), while the clusters III, IV, IX and XII included 4 strains. All the other clusters were smaller, including 1 or 2 strains. Based on the results of interdelta analysis, a strain, representative of each cluster, was further tested for its oenological traits.

3.1.2. Phenotypical characterization

The phenotypical characterization of the 13 autochthonous strains is reported in **Table 4**. Commercial wine yeast strains are primarily selected for their ability to ferment grape must to dryness, whereby residual sugars are reduced to less than 2 g l^{-1}. For a grape must to be fermented to dryness, the yeast strain is required to adapt and respond to a multitude of environmental stresses, either simultaneously or successively. The environmental stresses commonly encountered during commercial wine fermentations include a high initial sugar concentration, low nitrogen levels, and possible changes in the fermentation temperature, among others (Fairbairn 2012). Concerning sugar, all the

strains showed the same tolerance to glucose, fructose and sucrose (i.e. 300 g l^{-1}); this result is of concern because grape musts used in winemaking usually contain equal amounts of fructose and glucose in a range between 160 and 300 g l^{-1} (Tronchoni et al. 2009), while sucrose is by far the most abundant, cheap and important sugar in the industrial utilization of the yeast *S. cerevisiae* (Badotti et al. 2008). As regards ethanol, the strains exhibited a good resistance, as they were able to grow in media containing 18-20% of EtOH; this is an interesting trait, although it was not in accordance with previous studies carried out in Apulian region, as Cappello et al. (2004) and Tofalo et al. (2007) found that yeasts were inhibited by 14% and 15% of ethanol, respectively, but it is well known that *S. cerevisiae* shows a strong intra-species variability (Romano et al. 2008).

MIC for SO_2 was 300 ppm for the strains W41, W28, Y22 and W44, and 700 ppm for the strains Y25, W46, W48, Y10 and W13; this resistance appeared higher than that reported by Romano et al. (1998) and Fiore et al. (2005) for wild *S. cerevisiae* strains (125-150 and 300 ppm, respectively).

As regards copper resistance, all the strains showed MIC values from 500 to 900 ppm; two studies showed a similar trend: Shinohara et al. (2003), with many strains able to resist to 500 ppm and one strain to 700 ppm of $CuSO_4$, and Capece et al. (2010) with *ca.* 270 isolates able to grow in presence of 500 ppm of copper. The resistance to copper could be an advantageous trait for yeast selection, as Romano et al. (2004) reported that this trait is related to the ability of the strains to reduce copper concentration in the wine.

The high resistance of yeast strains to acetic acid (3.5 g l^{-1}) suggested a possible use in musts obtained from grapes infected by *Botrytis cinerea* or by Sour Rot, where acetic acid is 1 or 2 g l^{-1} (Vasserot et al. 2010); moreover the growth at 42°C could suggest a possible thermophilic behaviour (Castellari et al. 1998), similar to that recovered for some isolates of Mediterranean area, like Greece (Nikolaou et al. 2006) and Spain (Regodón et al. 1997). Finally, all the strains were able to grow under combined stress conditions, thus suggesting their suitability as wine starters.

Enzymes play an important role during winemaking. In particular, it is desirable for wine strains to possess β-D-glucosidase activity, as it is involved in flavour releasing processes (Strauss et al. 2001); therefore, this trait was assessed on four media containing different β-D-glucosidic substrates (arbutin, cellobiose, esculin and 4-MUG). Yeast strains exhibited a significant β-D-glucosidase activity only on the medium containing esculin

and this result is in agreement with Daenen et al. (2008), who reported that the majority of *Saccharomyces* isolates did not show β-D-glucosidase activity on a natural substrate like arbutin or cellobiose, and with Fiore et al. (2005), who found that *Saccharomyces* strains failed to exhibit β-D-glucosidase activity on 4-MUG substrate. β-D-glucosidase activity was strong for the strain W21, whereas the strain Y25 did not show any activity. The other strains showed this activity from weak to medium levels.

A topic of great concern for wine *S. cerevisiae* strains is the pectinase activity: 12 strains showed this activity at moderate level, whilst the strain W38 possessed a strong activity. Pectolytic enzymes are involved in pectin degradation, thus they could increase must-clarification rate, prevent the disappearance of important substances such as anthocyanins and the precursors of the varietal character, and avoid the use of physical approaches or the addition of commercial fungal pectinases for the clarification (Ubeda Iranzo et al. 1998).

The strains Y25, W43, W48, W38, W28, Y10, W13 and W3 showed a moderate xylanase activity, whilst the others possessed this trait at high level; xylanases could contribute to wine aroma by increasing the amount of monoterpenyldiglycoside precursors in the must. Moreover, hemicellulose could lead to filtration and clarification problems; the presence of xylanase activity in wine *S. cerevisiae* yeast can solve this problem (Strauss et al. 2001).

Concerning the other enzymatic activities, the results revealed that none of the strains showed protease, glycosidase, β-D-xilosidase and esterhydrolase (data not shown). These results were in agreement with the data reported by Strauss et al. (2001) and Comitini et al. (2011).

Finally, in order to establish how oenological conditions could affect the enzymatic potential of yeasts, β-D-glucosidase, pectolytic and xylanolitic activities were studied in some combinations that mimic the real conditions of wine (pH 3.5; ethanol 10%). Both pH and ethanol did not affect pectolytic and xylanolitic activities, while β-D-glucosidase activity was significantly reduced. These results confirmed some literature reports: Delcroix et al. (1994) found a 10% decrease in activity in 15% ethanol, whilst Mateo and Di Stefano (1997) described a stronger inhibitory effect of ethanol (5%) on β-D-glucosidase activity. Following other reports, β-D-glucosidase does not occur under the conditions of fermentation (Gunata et al. 1986) or its activity is largely limited at the pH of wine (Aryan et al. 1987).

Some additional phenotypical traits included the interaction with the phenolic compounds, hydrogen sulphide production and amino acid decarboxylation. Concerning the interaction with phenolic compounds, the strains showed a moderate ability to adsorb pigments from grape skins and seeds. The interaction with phenols is a new trend (Suárez-Lepe and Morata 2012), as a yeast could be selected for different traits and reasons: (i) the protection of colour during red winemaking; (ii) the removal of residual colour during white winemaking; (iii) the protection of phenolic compounds responsible for the antioxidant activity of wine (Caridi et al. 2007). As regards to hydrogen sulphide, this is one of the highly undesired compound produced by *S. cerevisiae* during fermentation, being associated with off-odors described as rotten egg and/or sewage (Cappello et al. 2004); the 13 strains produced low (Y25, W48 and W13) medium (W21, W46, W28, Y10, Y22 and W3) or high levels of this compound (W43, W41, W38 and W44). Finally yeasts were analyzed for their decarboxylase activity, potentially related to the presence of biogenic amines in the wine (Capece et al. 2012). None of the studied strains showed this activity (data not shown).

As a final step of the phenotyping, a Cluster Analysis determined whether the 13 strains could be grouped by their phenotypical properties. The statistical analysis produced the clusters reported in **Figure 2**. The strains were distributed in six main groups (from A to F): the group D included 5 different strains (W43, W3, W41, W38 and W44), characterized by a low level of dissimilarity or the same phenotypical profile (W43 and W3), whilst the other groups were smaller, containing 1, 2 o 3 strains.

3.1.3. Technological characterization

The autochthonous strains were used in some micro-fermentation trials to evaluate their main oenological characteristics; 3 commercial strains (EC1118, RC212 and BM45 from Lallemand Inc., Montreal, Canada) and a collection isolate of *S. cerevisiae* (DBVPG6500 from Industrial Yeasts Collection of the University of Perugia, Italy) were used as references (**Table 5**). The experiment was designed to be carried out at 25-30°C to fit better with the real conditions encountered in Apulian region; the fermentation of grape must is traditionally performed at the end of summer, during the warm days of September in the Mediterranean Countries at average temperatures between 25-30°C. These temperatures are also used by some producers to enhance the extraction of anthocyanin pigments (Belloch et al. 2008). Moreover, the experiments were performed

in media taking into account the typical sugar-composition of grape musts used in winemaking (Tronchoni et al. 2009). Under these controlled conditions the autochthonous *S. cerevisiae* showed: (i) high ethanol yields associated with an efficient conversion of grape sugars; (ii) a glycerol production exceeding 5.20 g l^{-1}, which is the threshold taste level of sweetness (Noble and Bursick 1984); (iii) volatile acidity concentrations < 1.2 g l^{-1} of acetic acid in almost all the strains, which is its legal limit according to European legislation (Vilela et al. 2013); (iv) amounts of residual sugar at levels below 2 g l^{-1} (data not shown). Data from small-scale fermentations were analyzed through one-way and two-way ANOVA, to highlight the significant differences amongst the different strains; the results are reported in the **Figure 3**. Concerning ethanol, the performances of the strains Y10 and Y22 were significantly higher than the commercial strains EC1118 and DBVPG6500. For glycerol, the strains DBVPG6500 and BM45 were the lowest and the highest producers, respectively. Finally, for the volatile acidity, the strains W21 and W48, as well as the reference strains (BM45 and EC1118), were the lowest producers.

3.1.4. OTA-removal and selection of the most promising strains

Amongst the 17 strains including the 4 commercial isolates (EC1118, RC212, BM45 and DBVPG6500), only 5 strains are able to remove ochratoxin A (OTA), namely W21, W46, W41, W28, W13 (**Table 6**). The table shows the percentage of OTA removal; the initial content of OTA was 2 μg l^{-1}, i.e. the legal limit in the European Union (Suárez-Lepe and Morata 2012). The bioremediation of the medium was assessed through ELISA, as Esti et al. (2012) used a kit similar to that proposed in this research and validated the method after a comparison with a HPLC-fluorescent approach; in addition, ELISA is a friendly and quick-protocol, if compared to the traditional HPLC-based approach.

The highest reduction of OTA was achieved with the strains W13 (from 36.58 to 48.63%), W28 (from 31.59 to 48.60%) and W46 (from 25.10 to 43.76%). These results confirmed that OTA removal was not a genus/species characteristic but a strain trait, as reported by Angioni et al. (2007).

As a final step of this experiment, we performed the selection of the most promising strains, using OTA removal as a primary selection tool. According to this characteristic, the strains W13, W28 and W46 showed the higher OTA-removal percentages coupled

with interesting phenotypical and technological traits including the ability to completely ferment sugars, a good ethanol and glycerol production, low acetic acid amounts, a high tolerance to single and combined fermentative stress conditions, β-D-glucosidase, pectolytic and xylanase activities, a low-to-medium level of hydrogen sulphide production, a low-to-medium parietal interaction with phenolic compounds and no potential biogenic amines formation; therefore, these strains were selected for the 2nd step of this research.

3.2. *IN VITRO* CHARACTERIZATION OF SELECTED STRAINS

The main goal of the second part of PhD thesis was to study 3 selected strains of *S. cerevisiae* for their fermentative performances and OTA-removing ability under *in vitro* conditions by modulating the time (0, 1, 3, 6 and 10 days), the temperature (25 and 30°C), sugar (200 and 250 g l^{-1}) and diammonium phosphate (DAP) (300 mg l^{-1}). The experiments took into account the YAN content normally ranging from 50 to 500 mg l^{-1} in grape must (Bely et al. 2003). On the other hand, nitrogen addition in form as DAP into nitrogen-deficient musts is a widely used practice to avoid fermentation problems such as sluggish or stuck fermentations (Henschke and Jiranek 1993).

Table 7 and **8** show the kinetics of the fermentation with the 3 selected strains in terms of cell level and residual sugar (**Table 7**), ethanol, glycerol and volatile acidity (**Table 8**).

These data were used as input values to run a multiple regression procedure; the statistical output of this approach is shown in **Table 9**.

3.2.1. Yeast growth and sugar consumption

All the strains reduced sugar content below the detection limit (2 g l^{-1}) and attained the highest level in cell count (*ca.* 8 log cfu ml^{-1}) after 3 days (**Table 7**).

As regard the statistical effects of time, temperature, sugar and DAP, yeast cell count was affected by the positive terms of temperature and sugars, i.e. cell count increased with increasing the sugar and the storage temperature, whilst DAP did not affect cell level; this latter result seemed to disagree with some literature reports (Henschke and Jiranek 1993; Bely et al. 2003). The effect of the time was significant for all the strains as a negative term. As expected the level of the residual sugars relied upon the initial concentration of the sugar and the time of sampling, respectively as positive and negative terms (**Table 9**).

3.2.2. Ethanol, glycerol and volatile acidity production

Temperature is a very important factor influencing ethanol production: for all the yeasts ethanol amount increased when temperature increased (**Table 9**) and this result was consistent with the effects reported by other authors (Fakruddin et al. 2012); moreover, ethanol production increased throughout the fermentation (effect of time) (**Table 9**). All the strains showed high ethanol yields associated to an efficient conversion of grape sugars; but the strain W13 produced the highest ethanol amounts (9.49-10.30 g l^{-1}), followed by the strain W46 (9.04-9.80 g l^{-1}) and W28 (7.93-9.04 g l^{-1}) (**Table 8**).

Glycerol production was positively affected by time and temperature, with the only exception of the strain W28, for which no correlation with temperature was found, but only a positive correlation with the initial sugar concentration (**Table 9**). Generally, a yeast responds to an increased sugar concentration with an enhanced production and intracellular accumulation of glycerol to counterbalance the osmotic pressure (Bely et al. 2008); moreover, glycerol increases as the temperature increases (Torija et al. 2003), and this effect was clearly recovered for the strains W13 and W46. On the other hand, none of the strains showed a positive correlation between DAP and glycerol. The different effects of environmental variables on glycerol production can be explained considering the significant phenotypic variation among winemaking strains (Hernández-Orte et al. 2006; Romano et al. 2008; Franco-Duarte et al. 2009). In general, all the strains showed a glycerol production higher than the threshold level (5.20 g l^{-1}) (Noble and Bursick 1984) with the strain W13 producing the highest glycerol amounts (7.22-8.55 g l^{-1}), followed by the strain W28 (5.69-6.91 g l^{-1}) and W46 (7.93-9.04) (**Table 8**).

Finally, the volatile acidity was positively affected by time and sugar, whilst DAP addition caused decreased levels in acetic acid (**Table 9**); similar findings have been already reported (Bely et al. 2003; Hernández-Orte et al. 2006). Regarding volatile acidity kinetics, the selected strains showed a similar trend, with a level of acetic acid lower than 1.2 g l^{-1} (**Table 8**) according to current European legislation (Vilela et al. 2013).

3.2.3. <u>In vitro</u> removal of OTA

Figure 4 shows the removal of OTA by the 3 selected strains throughout the time. The highest OTA removal was found for the strains W28 (3.94-74.92%) and W46 (2.82-73.09%). On the other hand, the strain W13 reduced OTA by 6.00 to 57.21%. Angioni

et al. (2007) reported that some *S. cerevisiae* strains were able to reduce OTA by 28.00-100.00% in an *in vitro* fermentation.

Concerning the effect of time, the removal was the highest one ($P<0.05$) after 3 days, then the toxin was partially released in the medium and the performances of the strains decreased; however, this process was random and the statistical analysis showed that the factors of the design (sugar, DAP and temperature) did not play any significant role (data not shown). The release of toxins after their removal was recovered for *Lb. amylovorus* and *Lb. rhamnosus* towards aflatoxin B_1 (AFB_1), due to the fact that the bonds between lactobacilli/AFB_1 complexes involve weak non-covalent interactions (Peltonen et al. 2001). A similar phenomenon could be associated to the release of OTA by *S. cerevisiae* since hydrogen bonding, ionic or hydrophobic interaction seem to be related to OTA adsorption mechanism (Cecchini et al. 2006). Moreover, OTA removal ability could be related to yeast kinetics as both OTA decrease and cell count attained the maximum level after 3 days and this result confirmed the correlation between cell count/OTA reported by Patharajan et al. (2011).

Regarding the environmental conditions on the removal of OTA, the multiple regression approach evidenced a positive effect of temperature and sugar (**Table 10**). In this sense, Patharajan et al. (2011) studied six different temperatures (10, 15, 20, 25, 30 and 35°C) to optimize OTA degradation by three yeasts (*Metschnikowia pulcherrima* MACH1, *Pichia guilliermondii* M8, *Rhodococcus erythropolis* AR14) and found the highest degradation at 30°C. It could be also suggested that the major ability to remove OTA at 30°C could be associated to the release of cell wall polysaccharides (Giovani et al. 2010). Concerning sugar concentration, our results confirmed the hypothesis reported by Meca et al. (2010): i.e. a yeast could show a higher performance in a must with a high sugar concentration than in a must obtained from dry grape, being the level of mannoproteins correlated to the level of sugars present in the medium.

3.3. *IN VIVO* CHARACTERIZATION OF SELECTED STRAINS

In the last part of this PhD project, the selected strains were characterized under *in vivo* conditions to investigate the ability to remove OTA during the fermentation of Uva di Troia red must and assess the role of temperature (25 and 30°C), sugar (200 and 250 g l^{-1}) and addition of DAP (300 mg l^{-1}) on this phenomenon; the stability of OTA-yeast complex was studied by evaluating the amount of toxin released back after some

washing treatments with phosphate-buffered saline (PBS, pH 3.5). Finally, the influence of OTA on the fermentation activity was investigated.

3.3.1. In vivo removal of OTA

The 3 strains were able to reduce the content of the toxin with percentages of removal of 30.69-76.44 (**Figures 5**-**7**). The strain W46 showed a kind of removal of 42.79-76.44%; on the other hand, the strains W28 and W13 were able to remove the toxin by 34.51 and 30.69 to 70.17 and 53.79%, respectively. Concerning the percentages of removal, the differences between the 3 targets were reported in **Figure 8**. OTA removal under *in vivo* conditions was reported by Piotrowska et al. (2013) for the strain Malaga LOCK 0173 capable to remove 82.80 and 10.70% OTA from grape and blackcurrant medium, respectively. On the other hand, Esti et al. (2012) reported the ability of two commercial yeast strains (Laffort-Zymaflor VL3 and Anchor-Lallemand VIN7) to reduce OTA (57.00-79.00%) during the alcoholic fermentation of red and white wine-must. Finally, Csutorás et al. (2013) showed the ability of a commercial *S. cerevisiae* type "Fermol Premier Cru" to remove OTA from red, rose and white must with reducing rates of 89.00, 85.00 and 75.00%, respectively.

Table 11 shows the statistical effect of temperature, sugar and DAP addition on OTA removal after the wine fermentation. The process was significantly affected by temperature, as reported elsewhere for the strains under *in vitro* conditions; namely, the ability to remove the toxin increased with increasing the temperature. As a general trend, the extent of removal of OTA increased with sugar content, for the strains W28 and W46; whilst, sugar content was not significant for the strain W13. DAP addition was significant for the strains W13 and W28; on the contrary, the effect was not significant under *in vitro* conditions. When DAP was added to the grape must, the YAN content increased from 114 to 174 mg l^{-1}; on the other hand, the YAN content of synthetic medium ranging from 250 to 310 mg l^{-1}. Our results suggest that the strains W13 and W28 exhibited a significant ability to remove OTA only under stressful conditions (i.e. lower YAN content). It could be suggested that OTA-removal could be associated to a greater release of cell wall mannoproteins, higher under a stress. Giovani et al. (2010) reported that the yeast cell wall is not a static entity; rather, it is dynamically remodelled in response to changes in environmental conditions. Indeed, cells can respond to environmental stress to varying degrees by changes in molecular architecture, changes in

the relationship between cell wall polysaccharides, and increases in the amounts of several cell wall proteins. As a consequence of this cellular adaptation response, the cell wall is remodelled in an attempt by the cell to survive.

A further explanation for the differences observed among the strains could be due to the fact that the removing of OTA is strictly strain-dependent (Angioni et al. 2007), thus we could suppose that the strain dependence plays a significant role also on the response to changes in environmental conditions.

As a final step, by combining the output of the 3D-plots (**Figures 5-7**) and the table of the statistical effects (**Table 11**), it could be inferred that the removal of the toxin was maximum when variables were set at the highest levels; nevertheless, temperature, sugar and DAP acted as single factors, as the interactive effect was not significant.

3.3.2. Study of OTA release by yeasts

Figure 9 shows the amount of OTA released back by yeasts after 3 consecutive washing treatments; generally, the strain W13 released the lowest amount of the toxin, with a total OTA released of *ca.* 55%, whilst the strains W28 and W46 released *ca.* 80-85% of the toxin.

The lower release of the toxin by *S. cerevisiae* W13 suggested a more stable complex cell wall/OTA for this yeast.

The release of OTA into the medium confirmed that the binding could be at least partially reversible, as suggested by the decrease of the removal ability reported for the AFB_1 by 12 strains of lactic acid bacteria (LAB) (Haskard et al. 2001). In addition, El-Nezami et al. (1996) demonstrated that AFB_1 binding by *L. rhamnosus* GG was partially reversible. This is in contrast to the removal of AFB_1 by *Flavobacterium aurantiacum* NRRLB-184, in which toxin associated with microorganism could not be recovered from the bacteria by washing with water or chloroform, or after sonic rupture of the bacteria. This is probably due to the different mechanism in AFB_1 removal by *Flavobacterium* (metabolic degradation) (Fazeli et al. 2009) opposite to the parietal adsorption of yeasts.

Finally, the amount of the toxin released back to the medium was used as dependent variable for a two-way ANOVA analysis; temperature, sugar and DAP addition were used as independent factors. As a result, only the kind of strain affected the binding stability of yeast-OTA complex (data not shown).

3.3.3. Wine fermentation performances and influence of OTA on fermentation activity

Table 12 shows the amount of ethanol, glycerol and volatile acidity produced by the 3 yeasts at the end of the fermentation of Uva di Troia. These results were used as input values for a multiple regression approach; the results of the statistical analysis are shown in the **Table 13**. A cell level of *ca.* 6 log cfu ml^{-1} and a sugar content below the detection limit (2 g l^{-1}) were also observed for all the strains (data not shown).

The temperature is a very important factor influencing ethanol production: for all the yeasts ethanol increased when temperature increased, as reported under *in vitro* conditions; moreover, sugar concentration affected as a negative term the ethanol yield of the strain W28. An interactive effect of temperature x sugar was observed for the strain W46 (**Table 13**). Generally, all the strains showed high ethanol yields associated to an efficient conversion of grape sugars, but the strain W13 produced the highest ethanol amounts (9.88-10.60 g l^{-1}) (**Table 12**).

Glycerol production was positively affected by sugar and temperature; moreover, a correlation with DAP was found only for the strain W13. The interactive effects temperature x DAP, and sugar x DAP, were observed for the strains W13 and W28, respectively (**Table 13**). A glycerol production higher than the threshold level (5.20 g l^{-1}) (Noble and Bursick 1984) was recovered; namely, the strain W13 produced the highest glycerol amounts (7.79-8.89 g l^{-1}) (**Table 12**).

Volatile acidity production was positively affected by temperature and sugar, whilst DAP addition caused decreased levels in acetic acid concentrations produced by the tested strains (**Table 13**); similar findings have been reported under *in vitro* conditions. Nevertheless, acetic acid production was lower than 1.2 g l^{-1} (**Table 12**) according to current European legislation (Vilela et al. 2013).

There are no significant differences in the amounts of ethanol, glycerol and volatile acidity, as well as in the cell count and residual sugar concentration between the media contaminated with OTA and without (data not shown) as reported by Meca et al. (2010) for the fermentation of Italian red wine Moscato; on the other hand Bizaj et al. (2009) recovered a detrimental effect of the toxin in synthetic media.

Chapter 4. CONCLUSIONS

4.1. SIGNFICANCE AND IMPACT OF PhD RESEARCH

Over the last few years, different studies were carried out on the selection of autochthonous strains of *Saccharomyces cerevisiae* as wine starters. Despite that native Uva di Troia red grapevine variety is commonly used as base wine for *controlled denomination of origin* (DOC) products in commercial wineries in the Apulian region (Southern Italy), no studies have been carried out to improve its enological characteristics through the use of indigenous strains of *S. cerevisiae*. Thus, in the present PhD thesis, we investigated on the enological potential of autochthonous *S. cerevisiae* strains isolated from Uva di Troia grape. Similarly, the researchers did not focus on the removal of ochratoxin A (OTA) as a possible tool to complement oenological characterization. In this PhD research, for the first time, the ability of *S. cerevisiae* to remove OTA during alcoholic fermentation was used as an additional trait in a yeast-characterization programme.

Moreover, in the past the yeasts used for OTA-removal assays were not intended as possible wine starters, whilst this PhD project is the first attempt to investigate wine strains of *S. cerevisiae* as functional starters (i.e. yeasts that show both the benefits of traditional starters and a health- or product-focused function). In this sense, the most promising strain (*S. cerevisiae* W13), selected amongst thirty-five autochthonous yeast isolates, showed the ability to remove OTA under *in vitro* (6.00-57.21%) and *in vivo* conditions (30.69-53.79%), fermenting completely sugars and producing high ethanol and glycerol content, low volatile acidity amount, as well as, showing a high tolerance to single and combined fermentative stress conditions, β-D-glucosidase, pectolytic and xylanase activities, a low level of hydrogen sulphide production, a low-to-medium parietal interaction with phenolic compounds and no potential biogenic amines formation. In addition, *S. cerevisiae* W13 showed the lowest release of OTA after its biological removal during the fermentation of Uva di Troia grape must. In this sense, this PhD project is the first report on the stability of OTA-yeast complex under winemaking conditions.

Finally, the novelty of this PhD thesis relies also upon the effect of the most important factors of wine fermentation (temperature, sugar and nitrogen concentration, nature of the yeast strain) on the complex phenomenon of OTA binding. The removal of the toxin was affected by the kind of strains and/or by the nutritional and environmental conditions.

4.2. FUTURE TRENDS

A future perspective could be a focus on the role of the charge of cell wall on the complex phenomena of OTA removal and release to minimize the amount of the toxin released back into the medium by the starter. In this sense, the application of a robust design (e.g. central composite design) could be useful to study the effects of different levels of sugar and DAP on both OTA removal and cell surface properties and build a general model able to predict yeast trend under different conditions.

Moreover, the OTA removal and release phenomena should be investigated in terms of the genetic determinants of cell wall properties in *S. cerevisiae* to help the design of strategies for the genetic improvement of potential functional starters.

Finally, the most promising strain (*S. cerevisiae* W13) should be used in a pilot-fermentation to assess its ability to compete with the natural microbiota of grape must.

TABLES

Table 1: Review of yeasts able to remove ochratoxin A (OTA) throughout wine fermentation*.

Strain	Type of assay	OTA removal (%)	References
Saccharomyces cerevisiae QA23	Red microvinification	68	Abrunhosa et al. (2005)
Saccharomyces sensu stricto:	White microvinification		Caridi et al. (2006)
1042, Sc226, Sc1661, Sc2489, Sc2717	Grape must naturally contaminated	40-58	
Sc708, Sc1304, Sc1483, Sc1766, Sc1864, Sc2621, Sc2640, TT77, TT254	Grape must naturally contaminated	61-75	
12233, Sc45, Sc254, Sc560, Sc2659, TT173	Grape must naturally contaminated	80-91	
1042, Sc226, Sc708, Sc1304, Sc1661 Sc1766, Sc2621, Sc2640, Sc2717, TT77, TT254	Grape must with OTA addition	68-79	
12233, Sc45, Sc254, Sc560, Sc1483, Sc1864, Sc2489, Sc2659, TT173	Grape must with OTA addition	80-83	
Saccharomyces cerevisiae, *Saccharomyces cerevisiae* x *Saccharomyces bayanus*, *Saccharomyces bayanus*, *Kloeckera apiculata*, *Torulaspora delbrueckii*, *Schizosaccharomyces pombe*, *Candida pulcherima*, *Saccharomycodes ludwigii*	White and red microvinification	47-70	Cecchini et al. (2006)
Saccharomyces cerevisiae QA23	Red vinification	68	Fernandes et al. (2007)
Saccharomyces cerevisiae	Red vinification	30	Grazioli et al. (2006)
Saccharomyces cerevisiae IOC R 9001	Red microvinification	41	Lasram et al. (2008)
Saccharomyces cerevisiae IOC R 9001	Rose microvinification	44	
Saccharomyces cerevisiae IOC R 9001	Red macrovinification	31	
Saccharomyces cerevisiae (16 strains: SCM1-SCM16)	Microvinification	33-50	Meca et al. (2010)
Saccharomyces uvarum, *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Kloeckera apiculata*, *Torulaspora delbrueckii*, *Schizosaccharomyces pombe*, *Candida pulcherima*, *Saccharomycodes ludwigii*	White and red vinification	40-72	Morassut et al. (2004)
Saccharomyces bayanus EC1118, *Saccharomyces cerevisiae* ICV-D80	Pilot scale vinification	77-100	Ponsone et al. (2009)

**Source:* Quintela et al. (2013).

Table 2: Experimental planning for the analytical determinations (residual sugars, ethanol, glycerol, volatile acidity and ochratoxin A; OTA) and cell count. The experiments were performed in duplicate over two different batches.

EXPERIMENTS	N° of strains	Time of sampling (day)						Variables						N° of samples
		0	1	3	6	10	E.F. †	Temperature (°C)		Sugar (g l^{-1})		DAP †† (mg l^{-1})		
								25	30	200	250	0	300	
Selection of autochthonous *Saccharomyces cerevisiae*	17*													272
In vitro characterization of selected strains	3													240
In vivo characterization of selected strains	3													96$+96

* including 4 commercial *S. cerevisiae* strains as references.
† E.F.: enf of fermentation.
†† DAP: diammonium phosphate.
$ samples without OTA.

Table 3: Classification of the results of phenotypical traits into qualitative codes. The codes were used as input values to run a Cluster Analysis and divide the strains in different groups. (1st part of PhD thesis).

Qualitative codes	0	1	2
Tolerance to ethanol (%)	/	18	20
Tolerance to sulphure dioxide (ppm)	300/400	600	700
Tolerance to copper (ppm)	500/600	700	900
Interaction with anthocian[a]	/	1	2
Interaction with tannin[a]	0	1	/
H_2S production[b]	1	2	3
Xylanase pH 6.0[c]	-	+	++
Xylanase pH 6.0 + 10% ethanol[c]	-	+	++
Pectinase pH 4.0[d]	-	+	++
β-D-glucosidase pH 6.0[e]	-	+/++	+++
β-D-glucosidase pH 6.0 + 10% ethanol[e]	-	+/++	+++

[a] Colour of biomass: '0' (white/pale grey; low adsorption of phenolic compounds); '1' (pale; medium adsorption); '2' (dark hazel; high adsorption).

[b] Colour of biomass: '0' (white; no hydrogen sulphide production); '1' (cream; low production); '2' (light brown; medium production); '3' (dark-brown; high production).

[c] Diameter of the halo: '-' (no halo); '+' (between 1–3 mm); '++' (>3 mm).

[d] Diameter of the growth zone: '-' (no growth); '+' (between 2–5.5 mm); '++' (>5.5 mm).

[e] Diameter of the halo on Esculin Glycerol Agar (EGA) medium: '-' (no growth); '+' weak activity (between 14–17 mm); '++' medium activity (18–22 mm); '+++' strong activity (≥ 23 mm).

Table 4: Phenotypical characterization of the strains of *Saccharomyces cerevisiae* representative of the genetic clusters.

	Y25	W21	W46	W43	W41	W48	W38	W28	Y10	Y22	W44	W13	W3
Resistance to single and combined oenological stress													
Single stress													
Glucose ($g\ l^{-1}$)	300	300	300	300	300	300	300	300	300	300	300	300	300
Fructose ($g\ l^{-1}$)	300	300	300	300	300	300	300	300	300	300	300	300	300
Sucrose ($g\ l^{-1}$)	300	300	300	300	300	300	300	300	300	300	300	300	300
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 42°C	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol (%)[a]	18	18	18	20	20	20	20	20	20	18	18	20	20
Acetic acid (3.5 $g\ l^{-1}$)	+	+	+	+	+	+	+	+	+	+	+	+	
Sulphur dioxide (ppm)[a]	700	600	700	400	300	700	400	300	700	300	300	700	400
Copper (ppm)[a]	600	500	600	600	500	900	500	600	900	900	600	700	600
Combined stress													
250 $g\ l^{-1}$ sucrose + 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+
10% ethanol + 150 ppm SO_2	+	+	+	+	+	+	+	+	+	+	+	+	+
10% ethanol + 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+
Enzymatic activities													
β-D-glucosidase[b]													
pH 6.0	-	+++	++	+	+	++	+	++	++	++	+	++	+
pH 6.0 + 10% of ethanol	-	++	++	-	-	++	-	++	++	+	-	++	-
pH 3.5	-	-	-	-	-	-	-	-	-	-	-	-	-
pH 3.5 + 10% of ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Pectinase[c]													
pH 4.0	+	+	+	+	+	+	++	+	+	+	+	+	+
pH 4.0 + 10% of ethanol	+	+	+	+	+	+	++	+	+	+	+	+	+
pH 3.5	+	+	+	+	+	+	++	+	+	+	+	+	+
pH 3.5 + 10% of ethanol	+	+	+	+	+	+	++	+	+	+	+	+	+
Xylanase[d]													
pH 6.0	+	++	++	+	++	+	+	+	+	++	++	+	+
pH 6.0 + 10% of ethanol	+	++	+	+	+	+	+	+	+	+	+	+	+
pH 3.5	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 3.5 + 10% of ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of undesirable compounds													
Hydrogen sulphide[e]	1	2	2	3	3	1	3	2	2	2	3	1	2
Interaction with phenolic compounds[f]													
Anthocian adsorption	1	1	1	1	1	1	1	1	1	2	1	1	1
Tannin adsorption	0	0	0	1	1	1	1	0	0	1	1	0	1

[a] Minimal Inhibitory Concentration (MIC).

[b] Diameter of the halo on Esculin Glycerol Agar (EGA) medium: '-' (no growth); '+' weak activity (between 14–17 mm); '++' medium activity (18–22 mm); '+++' strong activity (≥ 23 mm).

[c] Diameter of the growth zone: '-' (no growth); '+' (between 2–5.5 mm); '++' (>5.5 mm).

[d] Diameter of the halo: '-' (no halo); '+' (between 1–3 mm); '++' (>3 mm).

[e] Colour of biomass: '0' (white; no hydrogen sulphide production); '1' (cream; low production); '2' (light brown; medium production); '3' (dark-brown; high production).

[f] Colour of biomass: '0' (white/pale grey; low adsorption of phenolic compounds); '1' (pale; medium adsorption); '2' (dark hazel; high adsorption).

Table 5: Ethanol, glycerol and volatile acidity production under *in vitro* conditions by *Saccharomyces cerevisiae* strains representative of the genetic clusters. Mean values ± standard deviation. The experiments were carried out in a synthetic medium (pH 3.5) containing 2 μg l^{-1} of ochratoxin A (OTA).

Strain	**Ethanol (g l^{-1})**				**Glycerol (g l^{-1})**				**Volatile acidity (g l^{-1})†**			
	Sugar 200 g l^{-1}		**Sugar 250 g l^{-1}**		**Sugar 200 g l^{-1}**		**Sugar 250 g l^{-1}**		**Sugar 200 g l^{-1}**		**Sugar 250 g l^{-1}**	
	25°C	**30°C**	**25°C**	**30°C**	**25°C**	**30°C**	**25°C**	**30°C**	**25°C**	**30°C**	**25°C**	**30°C**
Y25	9.83±0.07	10.05±0.02	9.40±0.01	9.74±0.04	7.01±0.08	7.21±0.01	7.69±0.05	8.18±0.04	0.42±0.02	0.46±0.00	0.80±0.05	0.91±0.02
W21	9.98±0.02	10.30±0.02	9.53±0.00	9.83±0.05	6.74±0.09	6.90±0.04	7.61±0.02	8.17±0.05	0.28±0.09	0.32±0.01	0.47±0.02	0.52±0.02
W46	9.39±0.02	9.79±0.11	9.11±0.02	9.50±0.07	7.00±0.00	7.19±0.02	7.36±0.07	7.70±0.09	0.49±0.04	0.59±0.03	0.67±0.07	0.78±0.03
W43	9.67±0.05	9.96±0.02	9.09±0.02	9.40±0.06	6.33±0.17	6.67±0.14	7.62±0.12	8.07±0.05	0.86±0.01	0.93±0.00	1.07±0.12	1.25±0.02
W41	8.81±0.07	9.07±0.00	8.39±0.02	8.87±0.14	5.95±0.08	6.23±0.12	6.66±0.02	7.36±0.04	0.89±0.02	0.98±0.01	1.24±0.02	1.33±0.01
W48	10.25±0.03	10.58±0.00	9.66±0.16	10.06±0.06	6.96±0.07	7.16±0.05	7.77±0.09	8.26±0.07	0.32±0.01	0.38±0.00	0.49±0.09	0.58±0.08
W38	9.38±0.11	9.76±0.01	9.09±0.07	9.58±0.03	6.25±0.07	6.66±0.04	7.54±0.07	7.80±0.14	0.73±0.02	0.86±0.00	0.84±0.07	0.92±0.02
W28	8.61±0.00	9.08±0.04	8.09±0.02	8.65±0.05	5.84±0.11	6.13±0.12	6.37±0.07	6.68±0.04	0.41±0.02	0.49±0.05	0.56±0.06	0.68±0.04
Y10	10.47±0.06	10.7±0.00	10.28±0.07	10.49±0.00	6.17±0.08	6.58±0.04	7.43±0.07	7.75±0.05	0.38±0.03	0.42±0.01	0.64±0.07	0.73±0.06
Y22	10.58±0.06	10.75±0.01	9.95±0.09	10.21±0.10	7.15±0.08	7.59±0.04	8.00±0.08	8.58±0.04	0.40±0.03	0.45±0.00	0.67±0.08	0.76±0.04
W44	9.62±0.00	9.85±0.02	9.08±0.05	9.36±0.06	6.38±0.09	6.71±0.19	7.66±0.06	8.17±0.05	0.87±0.04	0.94±0.00	1.01±0.06	1.14±0.04
W13	9.82±0.01	9.94±0.04	9.43±0.07	9.55±0.03	7.24±0.05	7.66±0.07	7.64±0.04	7.85±0.08	0.49±0.05	0.58±0.04	0.66±0.07	0.78±0.04
W3	9.46±0.11	9.74±0.02	9.17±0.04	9.37±0.06	6.59±0.11	6.88±0.09	7.57±0.05	7.79±0.04	0.86±0.04	0.91±0.00	1.04±0.03	1.16±0.07
EC1118*	8.90±0.20	9.30±0.07	8.37±0.07	8.48±0.05	4.30±0.09	4.57±0.09	4.64±0.04	4.92±0.01	0.30±0.04	0.28±0.04	0.46±0.01	0.55±0.03
RC212*	9.82±0.01	10.13±0.01	10.30±0.08	9,99±0.02	5.81±0.04	6.19±0.05	6.50±0.07	6.72±0.07	0.69±0.02	0.59±0.02	0.83±0.05	0.77±0.05
BM45*	10.40±0.03	10.72±0.00	10.08±0.05	10.30±0.03	7.17±0.06	7.61±0.80	8.08±0.03	8.75±0.08	0.27±0.00	0.29±0.01	0.50±0.04	0.57±0.01
DBVPG6500*	8.35±0.15	8.58±0.18	8.07±0.04	8.38±0.07	3.19±0.20	3.98±0.24	3.57±0.04	4.61±0.07	0.80±0.04	0.69±0.02	0.95±0.02	1.00±0.02

† as acetic acid.
* reference strains.

Table 6: Ochratoxin A (OTA) removal (%) under *in vitro* conditions by the different *Saccharomyces cerevisiae* strains. Mean values ± standard deviation. The experiments were carried out in a synthetic medium (pH 3.5) containing 2 μg l^{-1} of OTA.

Strain	OTA-removal (%)			
	Sugar 200 g l^{-1}		Sugar 250 g l^{-1}	
	25°C	30°C	25°C	30°C
W21	0.54±0.00	13.93±0.78	20.95±1.48	34.45±2.01
W46	25.10±0.54	31.42±0.94	38.48±0.74	43.76±0.63
W41	10.56±0.80	24.58±0.72	20.95±1.48	34.45±2.01
W28	31.59±0.89	34.37±1.76	41.08±0.35	48.60±0.38
W13	36.58±1.06	39.58±1.76	46.08±0.35	48.63±0.35

Table 7: Sugar consumption and yeast count under *in vitro* conditions of *Saccharomyces cerevisiae* W13, W28 and W46 as a function of time, temperature, sugar concentration and supplementation with diammonium phosphate (DAP) (300 mg l^{-1}). Mean values ± standard deviation. The experiments were carried out in a synthetic medium (pH 3.5) containing 2 μg l^{-1} of ochratoxin A (OTA). When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 250 to 310 mg l^{-1}.

			W13				W28				W46			
	Time (d)	Sugar (g l^{-1})	25°C		30°C		25°C		30°C		25°C		30°C	
			Control	DAP	Control	DAP	Control	DAP	Control	DAP	Control	DAP	Control	DAP
R.S.† (g l^{-1})	1	200	158.45±2.19	156.00±1.41	153.00±1.41	149.00±1.41	185.40±1.97	168.55±5.30	175.85±3.04	159.90±1.83	171.45±6.43	156.25±1.76	158.00±5.65	141.50±4.94
		250	196.55±2.19	187.30±2.36	185.15±2.61	179.85±2.05	204.90±1.27	193.10±0.42	209.75±1.19	197.95±1.06	196.55±0.77	172.30±0.70	186.15±1.20	169.85±2.05
	3	200	79.32±0.95	66.50±2.12	74.00±2.82	59.00±1.41	89.20±2.54	72.65±1.06	80.15±4.31	64.41±0.86	70.32±2.36	56.50±2.12	64.00±2.82	49.00±1.41
		250	88.60±1.97	80.95±3.18	84.10±0.28	72.88±1.11	135.05±5.16	120.05±1.62	120.05±1.62	108.05±4.45	88.80±1.69	75.95±0.28	81.10±3.88	62.85±1.06
	6	200	19.55±0.63	9.56±0.15	15.40±0.84	5.08±0.45	43.65±1.90	23.60±2.54	30.97±3.57	19.80±1.97	30.55±2.05	17.58±2.70	25.40±0.84	12.50±2.96
		250	27.90±2.96	16.30±1.41	24.95±0.91	12.45±0.91	81.05±4.59	59.50±2.40	68.00±0.28	51.05±1.90	38.40±3.67	26.30±1.41	34.95±0.91	14.45±1.90
	10	200	N.D*	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
		250	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Yeast count (log cfu ml^{-1})$^{\$\$}$	1	200	7.69±0.09	7.80±0.11	7.78±0.03	7.90±0.01	7.68±0.08	7.71±0.12	7.76±0.06	7.74±0.02	7.72±0.10	7.71±0.12	7.78±0.03	7.90±0.01
		250	7.42±0.13	7.69±0.12	7.66±0.07	7.83±0.02	7.42±0.14	7.47±0.15	7.56±0.08	7.64±0.09	7.42±0.13	7.75±0.04	7.75±0.04	7.86±0.06
	3	200	8.67±0.05	8.79±0.05	8.79±0.05	8.84±0.02	7.90±0.02	7.92±0.02	7.94±0.01	7.94±0.00	8.73±0.02	7.92±0.02	8.81±0.08	8.89±0.05
		250	7.90±0.01	8.59±0.08	8.45±0.07	8.69±0.03	7.67±0.05	7.75±0.04	7.85±0.04	7.88±0.04	8.34±0.06	8.58±0.07	8.57±0.10	8.77±0.07
	6	200	7.48±0.22	7.69±0.12	7.66±0.07	7.83±0.07	5.77±0.04	5.72±0.05	5.74±0.01	5.72±0.08	7.64±0.00	5.72±0.05	7.75±0.04	8.16±0.33
		250	6.89±0.02	7.55±0.10	7.46±0.20	7.64±0.04	5.56±0.12	5.62±0.06	5.71±0.05	5.68±0.11	6.90±0.03	7.55±0.10	7.12±0.27	7.52±0.11
	10	200	5.85±0.03	5.91±0.03	5.92±0.04	6.46±0.17	5.74±0.02	5.73±0.03	5.72±0.02	5.73±0.03	5.86±0.04	5.73±0.03	5.91±0.02	6.56±0.03
		250	5.69±0.08	5.86±0.05	5.82±0.04	6.11±0.23	5.43±0.13	5.49±0.08	5.47±0.14	5.60±0.10	5.68±0.07	5.88±0.03	5.83±0.02	6.11±0.23

† R.S.: Residual sugars.
* Below the detection limit.
$^{\$\$}$ *Saccharomyces cerevisiae* W13, W28 and W46 were inoculated at 6.80, 6.64, and 6.80 log cfu ml^{-1}, respectively.

Table 8: Ethanol, glycerol and volatile acidity production under *in vitro* conditions by *Saccharomyces cerevisiae* W13, W28 and W46 as a function of time, temperature, sugar concentration and supplementation with diammonium phosphate (DAP) (300 mg l^{-1}). Mean values ± standard deviation. The experiments were carried out in a synthetic medium (pH 3.5) containing 2 μg l^{-1} of ochratoxin A (OTA). When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 250 to 310 mg l^{-1}.

			W13				W28				W46			
	Time (d)	Sugar (g l^{-1})	25°C		30°C		25°C		30°C		25°C		30°C	
			Control	DAP	Control	DAP	Control	DAP	Control	DAP	Control	DAP	Control	DAP
Ethanol (g l^{-1})	1	200	1.19±0.05	1.38±0.04	1.27±0.06	1.58±0.06	1.07±0.04	1.17±0.07	1.26±0.07	1.37±0.05	1.07±0.08	1.18±0.04	1.34±0.11	1.48±0.06
		250	1.02±0.01	1.24±0.04	1.14±0.04	1.41±0.00	0.84±0.07	0.93±0.05	1.17±0.08	1.27±0.07	1.02±0.01	1.09±0.02	1.14±0.04	1.61±0.04
	3	200	5.61±0.07	5.80±0.07	6.12±0.16	6.57±0.15	3.91±0.09	4.22±0.15	4.20±0.03	4.72±0.04	4.56±0.13	4.65±0.14	5.46±0.16	5.47±0.01
		250	5.19±0.05	5.39±0.02	5.61±0.07	5.83±0.07	2.99±0.16	3.00±0.04	3.99±0.02	4.06±0.06	4.19±0.05	4.39±0.02	4.96±0.13	4.93±0.21
	6	200	8.60±0.06	9.10±0.20	9.06±0.12	9.37±0.04	6.82±0.07	7.16±0.07	7.03±0.20	7.66±0.15	7.75±0.14	8.00±0.06	8.31±0.05	8.37±0.04
		250	8.30±0.03	8.61±0.09	8.51±0.04	8.72±0.01	6.26±0.17	6.40±0.10	6.65±0.03	6.53±0.05	7.50±0.03	7.61±0.09	8.31±0.04	8.37±0.05
	10	200	9.77±0.08	10.16±0.15	9.85±0.02	10.30±0.08	8.49±0.09	8.67±0.01	8.92±0.04	9.04±0.09	9.28±0.07	9.41±0.08	9.70±0.09	9.80±0.08
		250	9.49±0.09	9.82±0.06	9.62±0.04	9.94±0.04	7.93±0.06	8.05±0.05	8.52±0.09	8.51±0.02	9.04±0.02	9.22±0.07	9.57±0.02	9.74±0.04
Glycerol (g l^{-1})	1	200	1.08±0.04	1.29±0.12	1.20±0.07	1.35±0.07	0.87±0.14	1.00±0.01	1.27±0.05	1.33±0.07	1.18±0.04	1.24±0.04	1.40±0.07	1.48±0.04
		250	1.37±0.05	1.40±0.03	1.51±0.11	1.64±0.12	1.19±0.09	1.29±0.05	1.50±0.08	1.54±0.12	1.47±0.05	1.40±0.03	1.56±0.04	1.59±0.04
	3	200	3.09±0.11	3.58±0.19	3.55±0.14	3.77±0.14	2.49±0.09	2.63±0.12	2.87±0.15	2.92±0.15	3.45±0.02	3.53±0.12	3.90±0.07	3.92±0.07
		250	3.51±0.09	3.79±0.10	3.73±0.06	4.08±0.09	2.81±0.06	2.80±0.15	3.30±0.04	3.19±0.02	3.71±0.09	3.79±0.10	4.18±0.13	4.18±0.04
	6	200	6.30±0.10	6.77±0.16	6.91±0.15	7.42±0.17	4.22±0.15	4.27±0.08	4.53±0.12	4.62±0.07	5.30±0.10	5.62±0.09	5.91±0.15	5.98±0.23
		250	6.62±0.08	7.02±0.05	6.77±0.08	7.30±0.10	4.53±0.13	4.52±0.04	4.84±0.09	4.72±0.08	5.72±0.05	5.97±0.12	6.37±0.08	6.30±0.10
	10	200	7.22±0.16	7.58±0.04	7.75±0.07	8.14±0.19	5.69±0.03	5.80±0.12	6.04±0.09	6.12±0.14	7.01±0.17	7.08±0.04	7.37±0.05	7.44±0.09
		250	7.55±0.16	7.94±0.19	7.78±0.14	8.55±0.17	6.08±0.07	6.22±0.15	6.58±0.03	6.61±0.09	7.35±0.16	7.39±0.12	7.63±0.07	7.75±0.17
Volatile acidity (g l^{-1})*	1	200	0.13±0.01	0.11±0.00	0.20±0.00	0.17±0.01	0.19±0.02	0.20±0.01	0.24±0.02	0.19±0.02	0.29±0.02	0.25±0.04	0.35±0.03	0.25±0.01
		250	0.21±0.00	0.20±0.01	0.30±0.03	0.26±0.02	0.32±0.01	0.24±0.02	0.22±0.01	0.30±0.03	0.34±0.01	0.26±0.01	0.31±0.02	0.29±0.02
	3	200	0.29±0.02	0.22±0.01	0.34±0.04	0.27±0.05	0.29±0.02	0.27±0.01	0.39±0.02	0.33±0.00	0.40±0.04	0.35±0.03	0.49±0.02	0.34±0.02
		250	0.42±0.00	0.30±0.01	0.51±0.04	0.44±0.04	0.39±0.02	0.31±0.00	0.53±0.02	0.44±0.04	0.49±0.02	0.39±0.02	0.65±0.02	0.49±0.02
	6	200	0.43±0.04	0.33±0.03	0.49±0.02	0.40±0.02	0.39±0.02	0.33±0.04	0.48±0.01	0.41±0.00	0.49±0.02	0.43±0.02	0.51±0.02	0.39±0.02
		250	0.66±0.07	0.52±0.07	0.81±0.06	0.69±0.02	0.52±0.06	0.45±0.04	0.72±0.02	0.60±0.01	0.64±0.04	0.55±0.02	0.80±0.01	0.69±0.02
	10	200	0.51±0.02	0.36±0.02	0.59±0.02	0.42±0.01	0.39±0.00	0.34±0.04	0.49±0.02	0.42±0.01	0.48±0.04	0.42±0.01	0.52±0.01	0.44±0.02
		250	0.79±0.02	0.62±0.01	0.88±0.04	0.77±0.05	0.63±0.06	0.54±0.04	0.78±0.03	0.69±0.01	0.74±0.04	0.67±0.05	0.83±0.02	0.75±0.03

*as acetic acid.

Table 9: Statistical effects of time, temperature, sugar concentration and supplementation with diammonium phosphate (DAP) on cell count and fermentation products under *in vitro* conditions of *Saccharomyces cerevisiae* W13, W28 and W46. The experiments were carried out in a synthetic medium (pH 3.5) containing 200 or 250 g l^{-1} sugar, with or without DAP (300 mg l^{-1}) at 25 or 30°C. The medium was added with 2 μg l^{-1} of ochratoxin A (OTA). When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 250 to 310 mg l^{-1}. Multiple regression approach, forward method; *F*, Fisher test value; *ES*, standard error.

Model	Factors	Statistical effect		
		W13	**W28**	**W46**
Cell count	Time	-3.79	-6.53	-3.54
	Temperature	7.04	7.17	6.85
	Sugar	2.78	3.63	2.67
	DAP	-*	-	-
	Adjusted R^2	*0.982*	*0.983*	*0.981*
	F	*1103.70*	*1595.20*	*1048.60*
	Degrees of freedom	*4.76*	*3.77*	*4.76*
	ES	*0.98*	*0.86*	*1.07*
Residual sugars	Time	-18.86	-26.74	-18.17
	Temperature	-	-	-
	Sugar	4.67	8.45	4.60
	DAP	-	-	-
	Adjusted R^2	*0.917*	*0.964*	*0.914*
	F	*298.78*	*531.76*	*284.14*
	Degrees of freedom	*3.77*	*4.76*	*3.77*
	ES	*37.63*	*26.79*	*38.26*
Ethanol	Time	24.04	36.99	29.96
	Temperature	4.70	3.79	2.24
	Sugar	-	-2.83	-
	DAP	-	-	-
	Adjusted R^2	*0.954*	*0.978*	*0.969*
	F	*840.26*	*1206.60*	*828.37*
	Degrees of freedom	*2.78*	*3.77*	*3.77*
	ES	*1.38*	*0.76*	*1.06*
Glycerol	Time	29.66	40.89	32.51
	Temperature	3.50	-	6.76
	Sugar	-	7.62	-
	DAP	-	-	-
	Adjusted R^2	*0.968*	*0.983*	*0.975*
	F	*826.65*	*2385.90*	*1562.90*
	Degrees of freedom	*3.77*	*2.78*	*2.78*
	ES	*0.88*	*0.47*	*0.72*
Volatile acidity	Time	14.66	12.13	11.75
	Temperature	-	-	-
	Sugar	3.29	7.24	7.99
	DAP	-2.99	-1.99	-2.40
	Adjusted R2	*0.912*	*0.901*	*0.903*
	F	*208.36*	*242.67*	*248.25*
	Degrees of freedom	*4.76*	*3.77*	*3.77*
	ES	*0.12*	*0.12*	*0.14*

*-, not significant.

Table 10: Statistical effects of time, temperature, sugar concentration and supplementation with diammonium phosphate (DAP) on the removal of ochratoxin A (OTA) under *in vitro* conditions by *Saccharomyces cerevisiae* W13, W28 and W46. The experiments were carried out in a synthetic medium (pH 3.5) containing 200 or 250 g l^{-1} sugar, with or without DAP (300 mg l^{-1}) at 25 or 30°C. Medium was added with 2 μg l^{-1} of OTA. When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 250 to 310 mg l^{-1}. Multiple regression approach, forward method; *F*, Fisher test value; *ES*, standard error.

Model	Factors	Statistical effect		
		W13	**W28**	**W46**
OTA removal	Time	-	-	-
	Temperature	5.86	3.28	3.96
	Sugar	2.50	3.82	4.06
	DAP	-	-	-
	Adjusted R^2	*0.978*	*0.970*	*0.977*
	F	*1230.80*	*874.54*	*1132.40*
	Degrees of freedom	*3.77*	*3.77*	*3.77*
	ES	*7.94*	*9.46*	*7.86*

*-, not significant.

Table 11: Statistical effects of temperature (1), sugar (2) and supplementation with diammonium phosphate (DAP) (3) on the removal of ochratoxin A (OTA) under *in vivo* conditions by *Saccharomyces cerevisiae* W13, W28 and W46. The experiments were carried out in a red grape must (pH 3.5) containing 200 or 250 g l^{-1} sugar, with or without DAP (300 mg l^{-1}) at 25 or 30°C. Medium was added with 2 μg l^{-1} of OTA. When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 114 to 174 mg l^{-1}. (1 by 2, interactive effect of temperature/sugar; 1 by 3, temperature/DAP; 2 by 3, sugar/DAP). The statistical analysis was performed through the option DoE of the software STATISTICA for WINDOWS.

Model	**Factors**	**Statistical effect**		
		W13	**W28**	**W46**
OTA removal	(1)Temperature	5.26	7.04	8.68
	(2)Sugar	-	6.48	3.05
	(3)DAP	6.92	6.37	-
	1 by 2	-	-	-
	1 by 3	-	-	-
	2 by 3	-	-	-
	Adjusted R^2	*0.828*	*0.897*	*0.849*

*-, not significant.

Table 12: Ethanol, glycerol and volatile acidity production under *in vivo* conditions by *Saccharomyces cerevisiae* W13, W28 and W46 as a function of temperature, sugar concentration and supplementation with diammonium phosphate (DAP) (300 mg l^{-1}). Mean values ± standard deviation. The experiments were carried out in a red grape must (pH 3.5), with or without ochratoxin A (OTA) (2 μg l^{-1}). When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 114 to 174 mg l^{-1}.

			W13				**W28**				**W46**			
	OTA	**Sugar (g l^{-1})**	**25°C**		**30°C**		**25°C**		**30°C**		**25°C**		**30°C**	
			Control	**DAP**	**Control**	**DAP**	**Control**	**DAP**	**Control**	**DAP**	**Control**	**DAP**	**Control**	**DAP**
Ethanol (g l^{-1})	-	200	9.95±0.07	10.41±0.08	10.09±0.02	10.60±0.08	8.79±0.12	8.94±0.05	9.28±0.07	9.39±0.07	9.60±0.07	9.78±0.12	10.03±0.05	10.03±0.03
	+	200	9.95±0.03	10.41±0.04	10.06±0.02	10.59±0.02	8.79±0.14	8.94±0.00	9.32±0.02	9.39±0.02	9.63±0.06	9.79±0.15	10.01±0.01	10.06±0.02
	-	250	9.89±0.09	10.42±0.07	10.02±0.09	10.43±0.05	8.29±0.01	8.44±0.06	8.90±0.14	8.84±0.06	9.42±0.09	9.72±0.07	10.05±0.14	10.26±0.00
	+	250	9.88±0.13	10.44±0.04	10.01±0.04	10.42±0.01	8.32±0.02	8.47±0.05	9.01±0.04	8.88±0.02	9.39±0.01	9.67±0.05	9.99±0.07	10.27±0.04
Glycerol (g l^{-1})	-	200	7.58±0.04	7.95±0.07	7.96±0.06	8.39±0.01	5.86±0.08	6.04±0.09	6.27±0.07	6.41±0.01	7.22±0.15	7.39±0.01	7.68±0.03	7.84±0.05
	+	200	7.60±0.01	7.96±0.02	7.99±0.01	8.35±0.06	5.88±0.13	6.11±0.11	6.32±0.05	6.39±0.01	7.25±0.12	7.34±0.08	7.65±0.06	7.85±0.06
	-	250	7.83±0.04	8.19±0.12	8.15±0.06	8.89±0.12	6.38±0.07	6.46±0.09	6.95±0.07	6.82±0.08	7.63±0.04	7.79±0.12	8.04±0.06	7.92±0.02
	+	250	7.79±0.02	8.20±0.02	8.08±0.07	8.85±0.04	6.39±0.02	6.48±0.14	6.96±0.02	6.81±0.00	7.62±0.02	7.79±0.04	8.02±0.02	7.92±0.07
Volatile acidity (g l^{-1})*	-	200	0.58±0.04	0.39±0.01	0.63±0.03	0.45±0.05	0.48±0.06	0.37±0.04	0.57±0.04	0.48±0.04	0.57±0.01	0.48±0.00	0.70±0.03	0.56±0.02
	+	200	0.59±0.05	0.38±0.02	0.65±0.01	0.47±0.05	0.52±0.05	0.38±0.01	0.61±0.03	0.50±0.03	0.57±0.02	0.47±0.05	0.74±0.04	0.55±0.02
	-	250	1.00±0.07	0.67±0.04	1.08±0.03	0.93±0.04	0.81±0.02	0.55±0.07	1.02±0.04	0.70±0.03	0.94±0.04	0.70±0.02	1.09±0.01	0.82±0.03
	+	250	1.01±0.04	0.65±0.03	1.10±0.02	0.95±0.01	0.82±0.02	0.55±0.03	1.03±0.01	0.70±0.01	0.95±0.01	0.68±0.02	1.07±0.04	0.82±0.01

*as acetic acid.

Table 13: Statistical effects of temperature (1), sugar (2) and supplementation with diammonium phosphate (DAP) (3) on ethanol, glycerol and volatile acidity production under *in vivo* conditions by *Saccharomyces cerevisiae* W13, W28 and W46. The experiments were carried out in a red grape must (pH 3.5) containing 200 or 250 g l^{-1} sugar, with or without DAP (300 mg l^{-1}) at 25 or 30°C. Medium was added with 2 μg l^{-1} of ochratoxin A (OTA). When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 114 to 174 mg l^{-1}. (1 by 2, interactive effect of temperature/sugar; 1 by 3, temperature/DAP; 2 by 3, sugar/DAP). The statistical analysis was performed through the option DoE of the software STATISTICA for WINDOWS.

Model	**Factors**	**Statistical effect**		
		W13	**W28**	**W46**
Ethanol	(1)Temperature	2.87	11.45	11.12
	(2)Sugar	-	-11.27	-
	(3)DAP	12.03	-	4.23
	1 by 2	-	-	2.96
	1 by 3	-	-	-
	2 by 3	-	-	-
	Adjusted R^2	*0.910*	*0.945*	*0.909*
Glycerol	(1)Temperature	10.00	10.85	8.34
	(2)Sugar	6.46	12.88	7.20
	(3)DAP	10.33	-	-
	1 by 2	-	-	-
	1 by 3	2.31	-	-
	2 by 3	-	-2.34	-
	Adjusted R^2	*0.943*	*0.950*	*0.896*
Volatile acidity	(1)Temperature	4.42	5.56	8.72
	(2)Sugar	15.91	11.88	22.21
	(3)DAP	-8.32	-7.87	-13.21
	1 by 2	-	-	-
	1 by 3	-	-	-
	2 by 3	-	-3.76	-5.12
	Adjusted R^2	*0.958*	*0.942*	*0.980*

*-, not significant.

FIGURES

Figure 1: Amplification of interdelta region patterns of thirty-five *Saccharomyces cerevisiae* isolated from 'Uva di Troia' grape and UPGMA (unweighted pair group method with arithmetic mean) clustering analysis for interdelta patterns. Strain grouping in different clusters (from I to XIII) (*strain selected as representative of each cluster to assess the phenotypical, technological properties, and ochratoxin A-removal ability, OTA); ($ Accession Numbers).

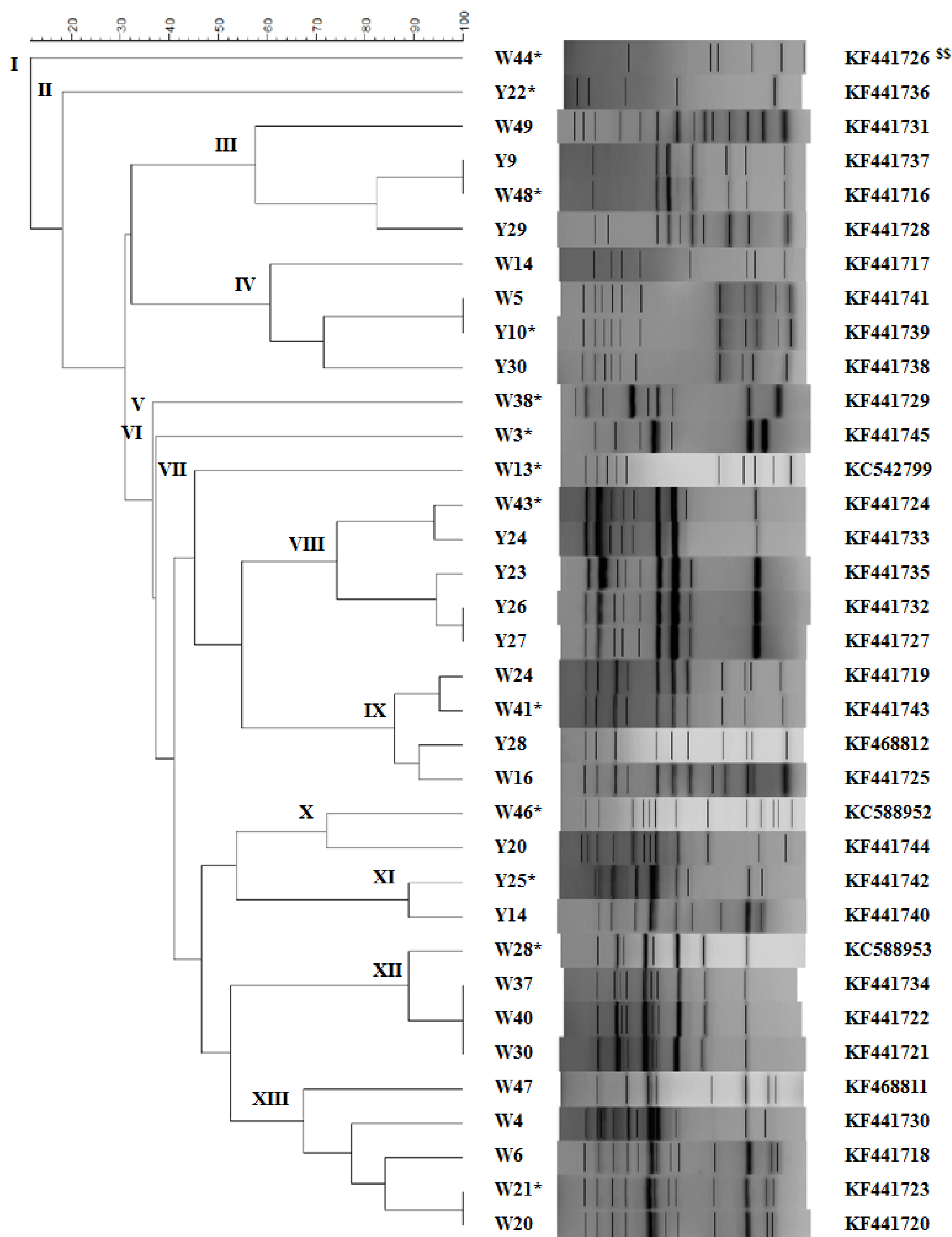


Figure 2: Classification of the *Saccharomyces cerevisiae* strains into different statistical groups through the cluster analysis. The statistic was performed by using the qualitative codes from the phenotypical traits (for the input variables see Table 3).

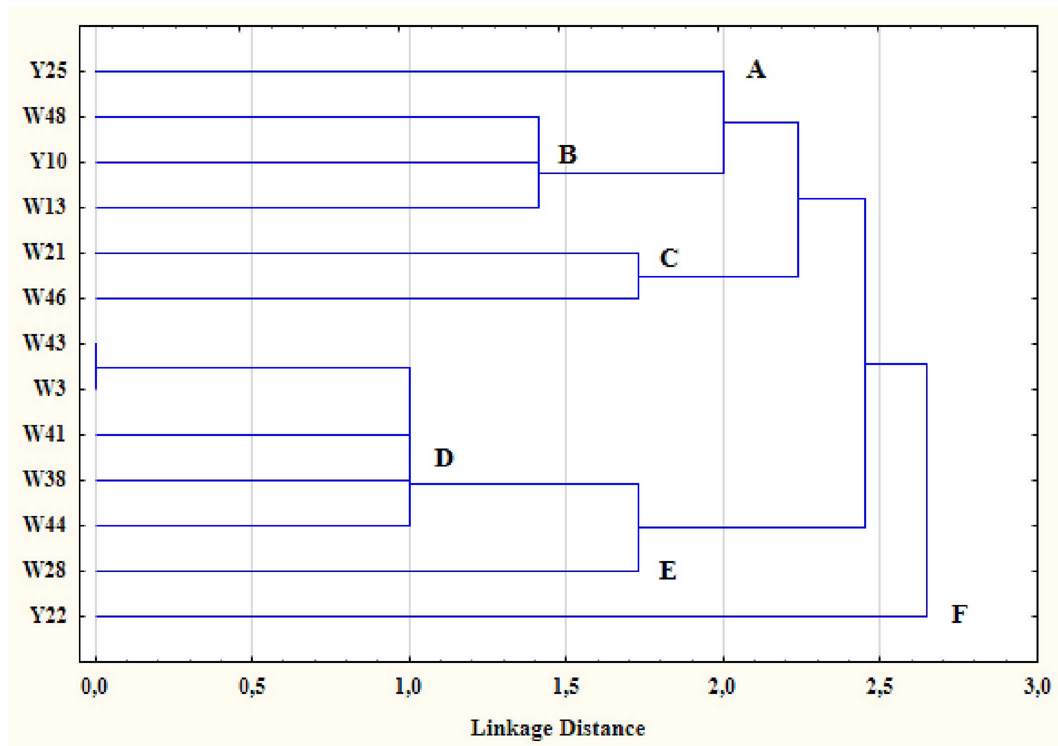


Figure 3: Effective decomposition of two-way ANOVA: effects of the strains of *Saccharomyces cerevisiae* on the production of ethanol, glycerol and volatile acidity. The experiments were carried out in a synthetic medium (pH 3.5) containing 200 or 250 g l^{-1} sugar at 25 or 30°C. Medium was added with 2 μg l^{-1} of ochratoxin A (OTA). *, reference strains

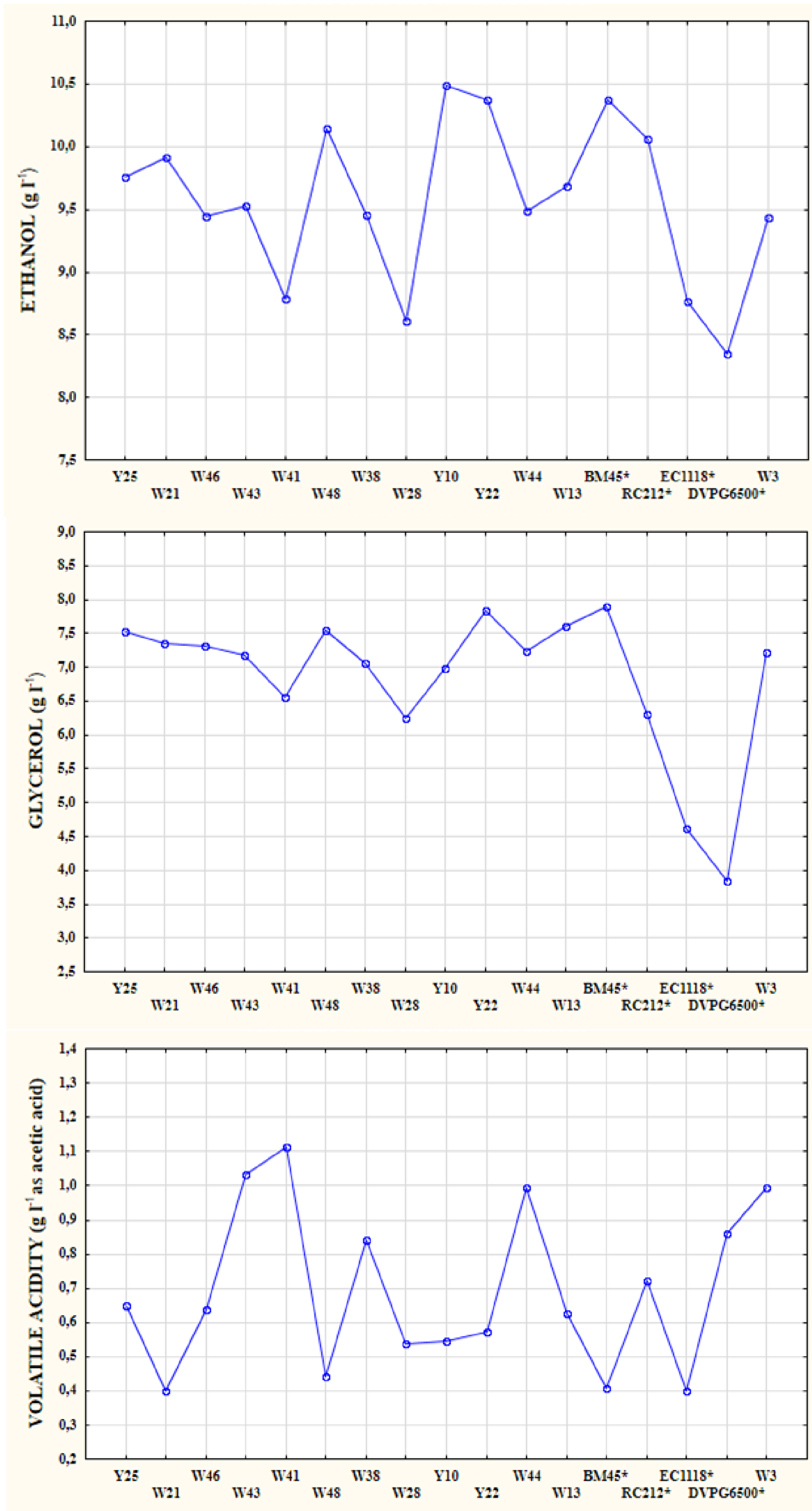


Figure 4: Ochratoxin A (OTA) removal (%) under *in vitro* conditions by *Saccharomyces cerevisiae* W13 (a), W28 (b) and W46 (c) as a function of time (1, 3, 6 and 10 days), temperature (25 and 30°C), sugar concentration (200 and 250 g l^{-1}) and supplementation with diammonium phosphate (DAP) (300 mg l^{-1}). Mean values±standard deviation. The experiments were carried out in a synthetic medium (pH 3.5) added with 2 μg l^{-1} of OTA. When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 250 to 310 mg l^{-1}.

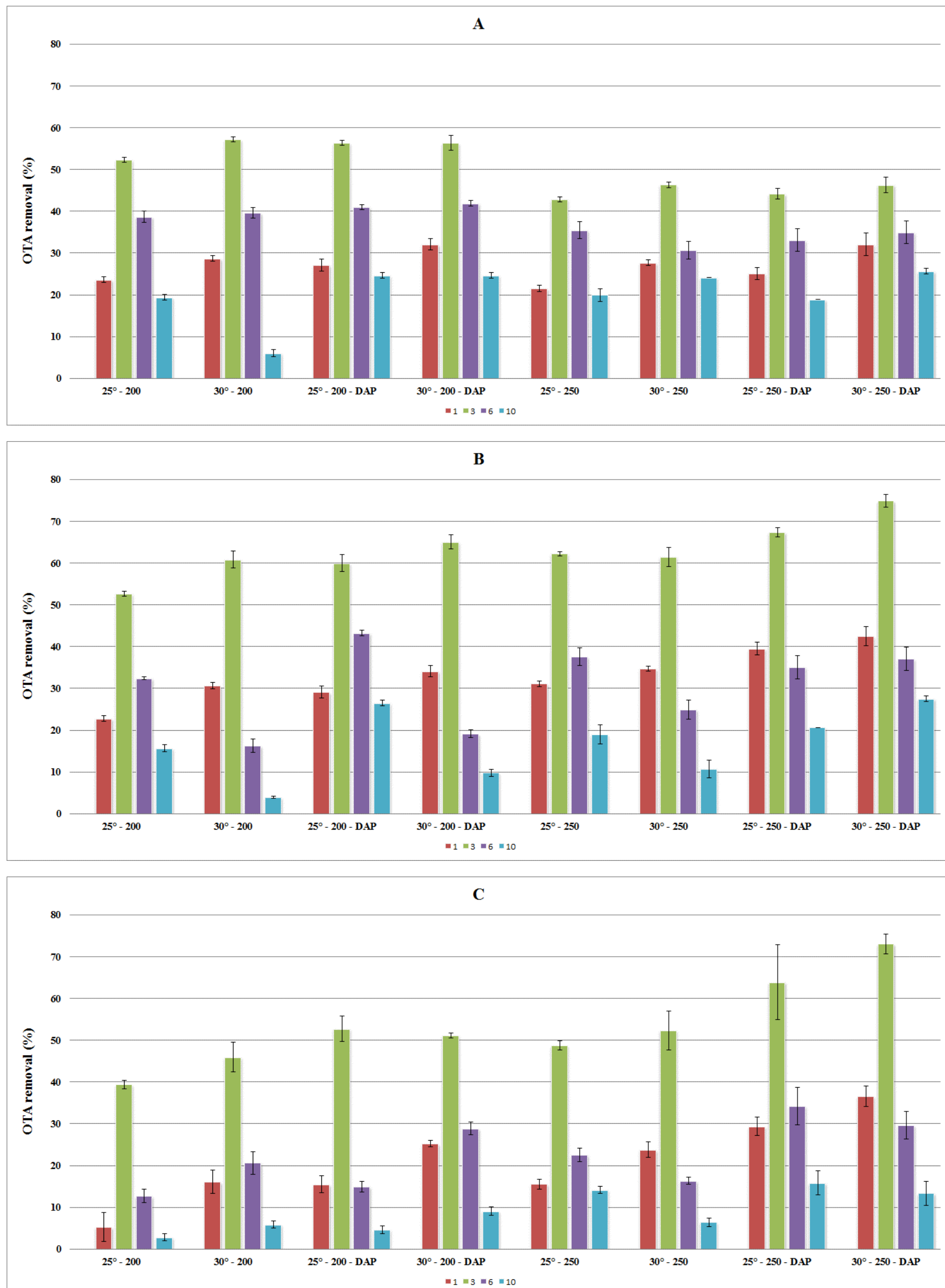


Figure 5: Three-dimensional plot for the interaction of temperature x diammonium phosphate (DAP) on ochratoxin A (OTA) removal ability under *in vivo* conditions by *Saccharomyces cerevisiae* W13. The experiments were carried out in a red grape must (pH 3.5) added with 2 μg l^{-1} of OTA.

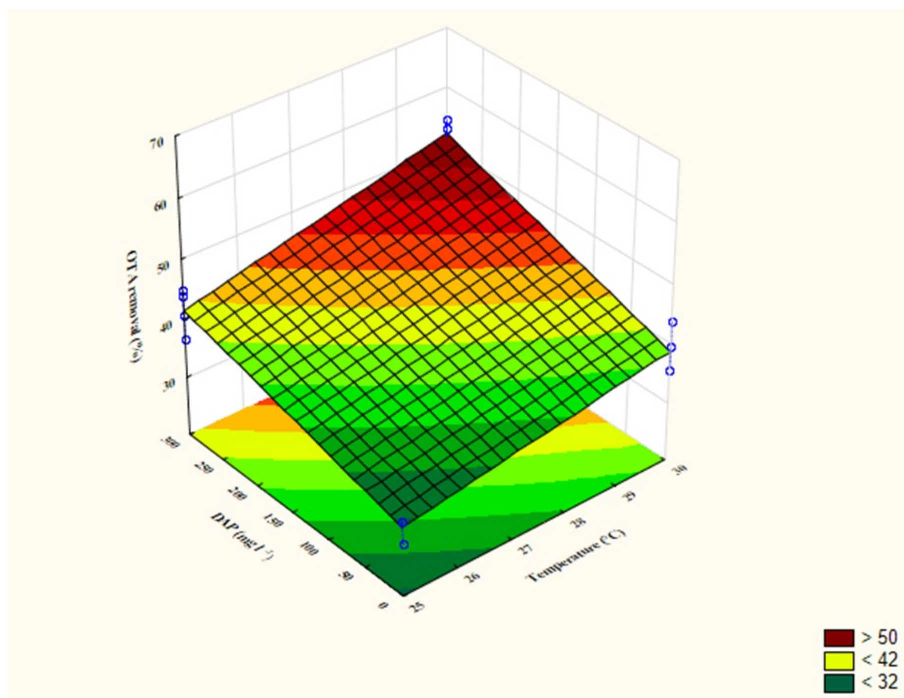


Figure 6: Three-dimensional plot for the interaction of temperature x sugar (a), temperature x diammonium phosphate (DAP) (b) and sugar x DAP (c) on ochratoxin A (OTA) removal ability under *in vivo* conditions by *Saccharomyces cerevisiae* W28. The experiments were carried out in a red grape must (pH 3.5) added with 2 μg l^{-1} of OTA.

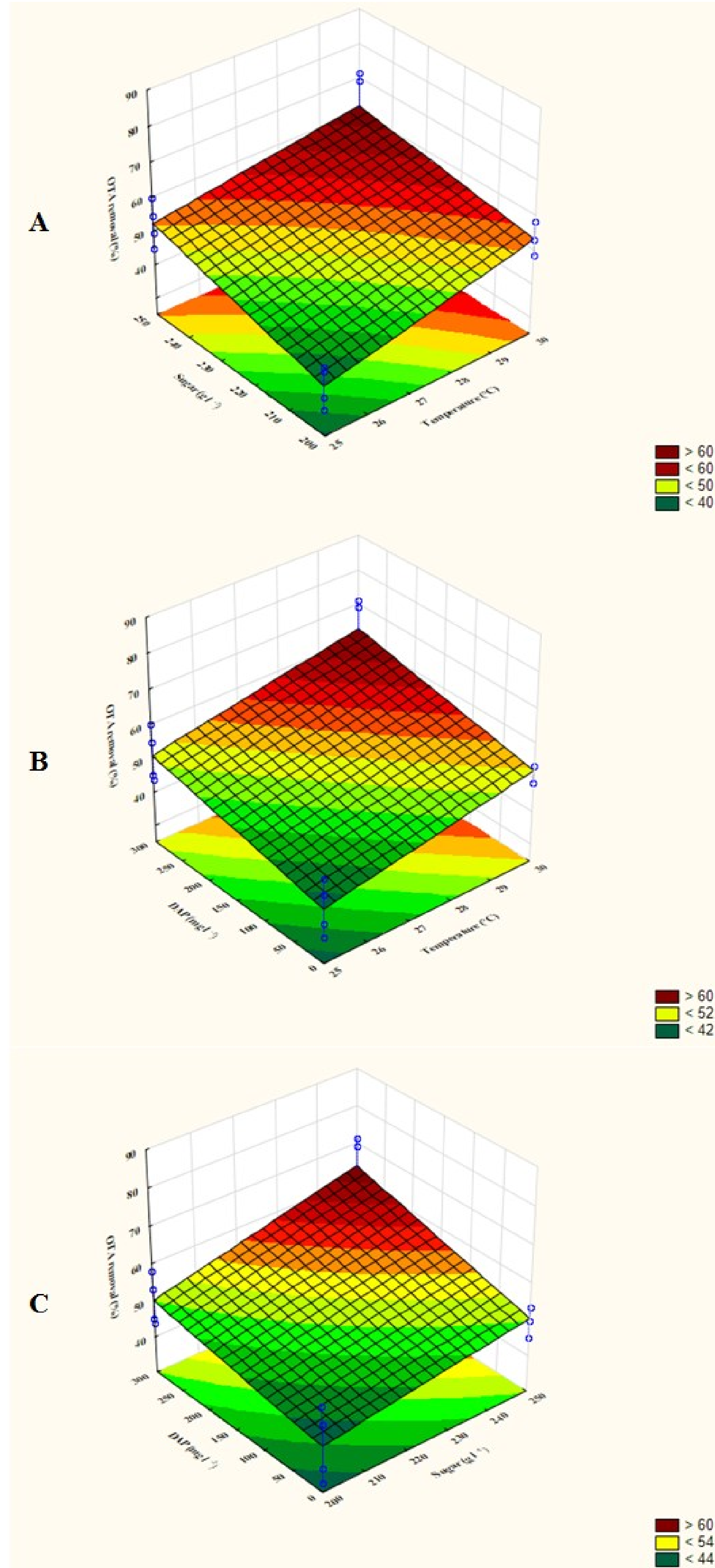


Figure 7: Three-dimensional plot for the interaction of temperature x sugar on ochratoxin A (OTA) removal ability under *in vivo* conditions by *Saccharomyces cerevisiae* W46. The experiments were carried out in a red grape must (pH 3.5) added with 2 μg l^{-1} of OTA.

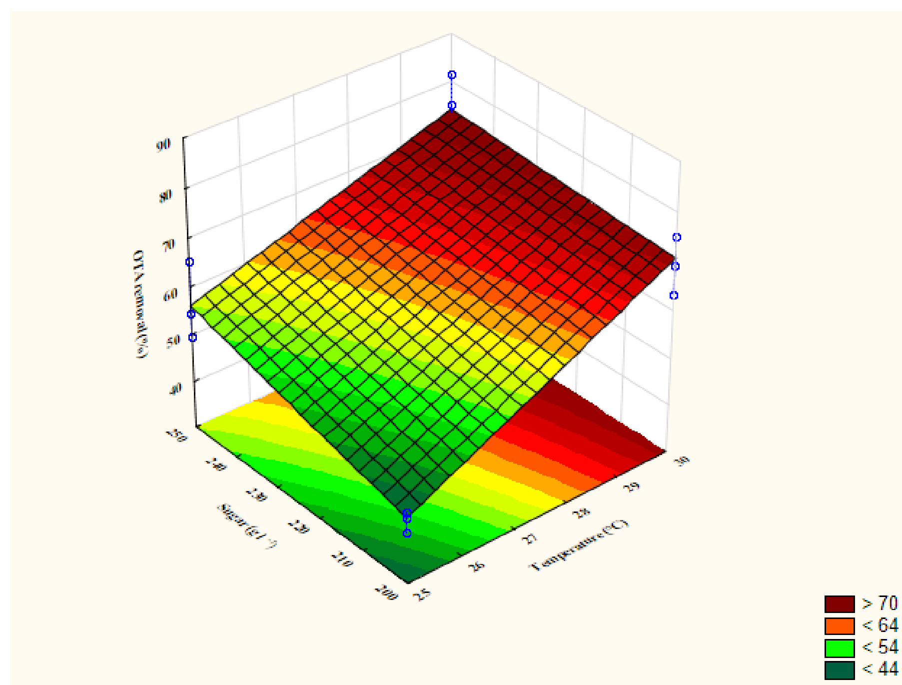


Figure 8: Two-way ANOVA for the effects of the strain on the amount of ochratoxin A (OTA) removed under *in vivo* conditions by *Saccharomyces cerevisiae* W13, W28 and W46. The experiments were carried out in a red grape must (pH 3.5) containing 200 or 250 g l^{-1} sugar, with or without diammonium phosphate DAP (300 mg l^{-1}) at 25 or 30°C. The medium was added with 2 μg l^{-1} of OTA. When DAP was added to the medium, the yeast assimilable nitrogen (YAN) content increased from 114 to 174 mg l^{-1}. The vertical bars denote 95%-confidence intervals.

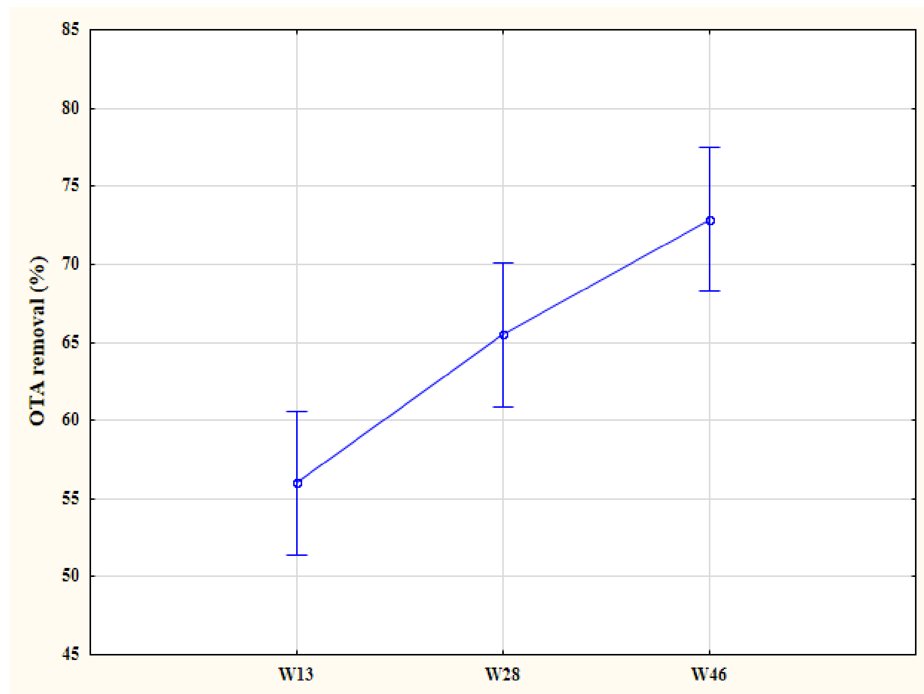
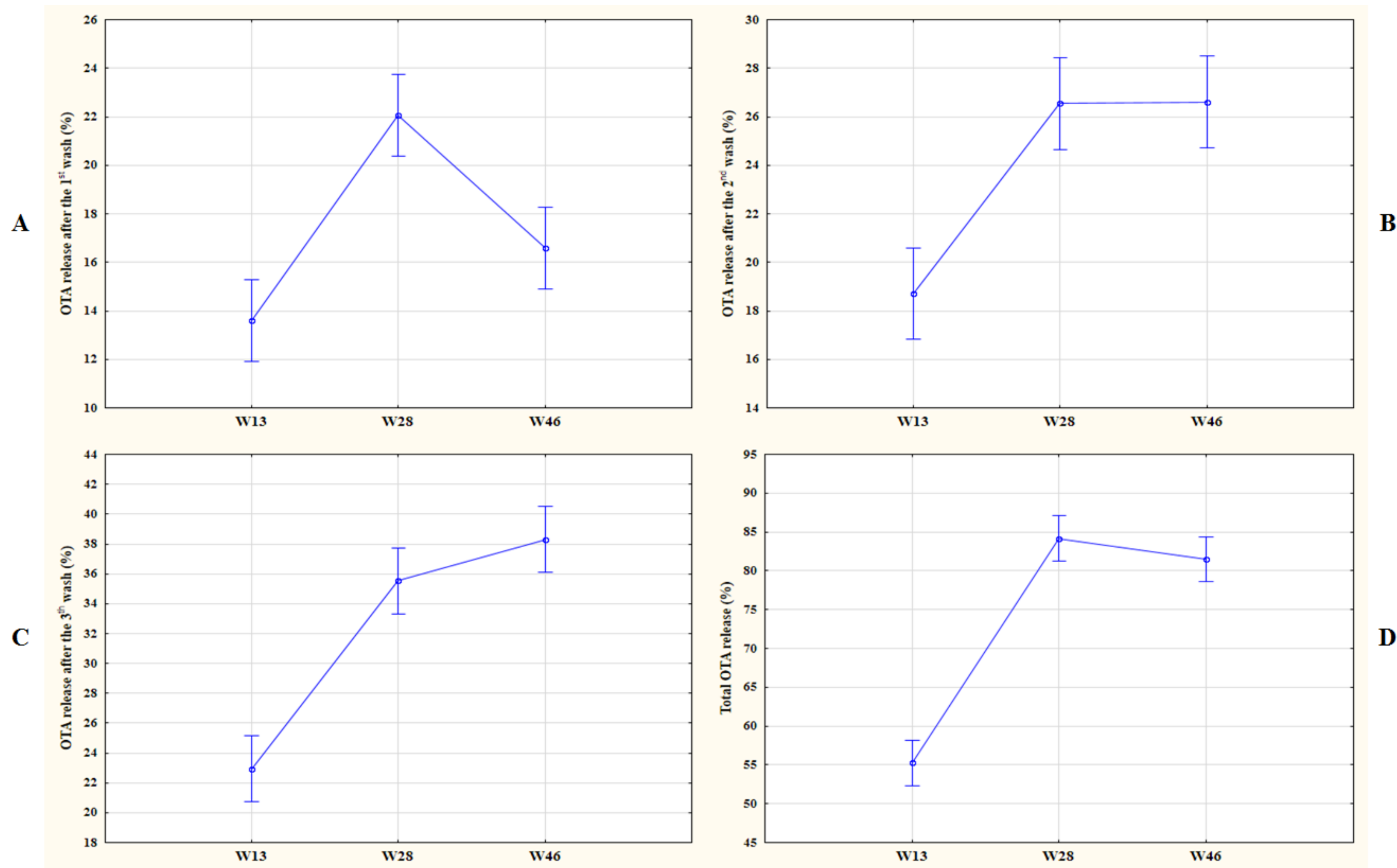


Figure 9: Two-way ANOVA for the effects of the strain on the amount of ochratoxin A (OTA) released back after 3 consecutive washing treatments (a, b, c) and total OTA (d) released back by *Saccharomyces cerevisiae* W13, W28 and W46. The *Saccharomyces*/OTA complexes formed under *in vivo* conditions were subjected to 3 washes with phosphate-buffered saline (PBS, pH 3.5) and the amount of OTA released was quantitated. The vertical bars denote 95%-confidence intervals.



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APPENDIX

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Study of *Saccharomyces cerevisiae* W13 as a functional starter for the removal of ochratoxin A

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In this paper, a wild strain of *Saccharomyces cerevisiae* (W13) was studied for its OTA-removal ability, growth pattern and alcoholic fermentation profile at two different temperatures (25 and 30 °C) and two different sugar levels (200 and 250 g l^{-1}), with or without supplementation of medium with diammonium phosphate (DAP). A commercial strain (Lallemand EC1118) and a collection isolate (DBVPG 6500) of *S. cerevisiae* were also studied. All the strains were able to conclude fermentation, with *S. cerevisiae* W13 producing higher ethanol and glycerol contents; moreover, the wild strain was able to remove OTA (reduction of 6–57.21%), with the highest removing effect observed at 30 °C with 250 g l^{-1} sugar and after 3 days.

In addition, *S. cerevisiae* W13 was studied for its technological and qualitative traits, as it showed a high tolerance to single and combined stress conditions, β-D-glucosidase, pectolytic and xylanase activities, a low level of hydrogen sulphide production, a low-to-medium parietal interaction with phenolic compounds and no biogenic amines formation.

The findings of this work have an applicative value for the potentiality of using *S. cerevisiae* W13 as functional starter for the production of wines with improved qualitative and food safety characteristics.

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1. Introduction

The technological and qualitative key-traits for the selection of a wine starter are the tolerance and high ethanol production, the exhaustion of sugars, the growth at high sugar concentration, a good glycerol production, the growth at high temperatures, a low hydrogen sulphide and volatile acidity production, the resistance to sulphur dioxide, and a good enzymatic profile (Nikolaou, Soufleros, Bouloumpasi, & Tzanetakis, 2006).

Functional starter cultures represent a new frontier goal for the design of food products, as they show both the benefits of traditional starters and a health- or product-focused function (Bevilacqua et al., 2012). A particular kind of functional trait for wine yeasts could be the removal of ochratoxin A (OTA); this trait has received a special focus, as this mycotoxin is considered the principal safety hazard in the winemaking process (Delage, d'Harlingue, Colonna Ceccaldi, & Bompeix, 2003). Many authors considered the use of yeasts as an effective tool to remove OTA both in a synthetic and in a natural grape juice (Bejaoui, Mathieu, Taillandier, & Lebrihi, 2004), in white and red musts (Caridi, Galvano, Tafuri, & Ritieni, 2006; Cecchini, Morassuta, Moruno, & Di Stefano, 2006), and model wine (Nunez, Pueyo, Carrascosa, & Martínez-Rodríguez, 2008). The biological approaches for mycotoxin removing are considered as promising solutions, since it is possible to attain the decontamination without using harmful chemicals and without losses in nutrient value or palatability of decontaminated food (Anly & Bayram, 2009).

A drawback in the literature is that few data are available on the oenological traits of OTA-removing yeasts, whilst this aspect is of great concern for the possible selection of strains acting at the same time as starters and as biological tools to remove the toxin. Therefore, the main aim of this work was to propose a wild strain of *Saccharomyces cerevisiae* (*S. cerevisiae* W13), able to remove OTA, as a potential starter. This goal was achieved through some intermediate scientific aims, i.e. by assessing the technological performances of the wild strain under various conditions and comparing them with the traits of two commercial strains; thereafter by studying the oenological traits of the proposed strain and its ability to remove OTA in a model system.

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ORIGINAL ARTICLE

In vitro removal of ochratoxin A by two strains of *Saccharomyces cerevisiae* and their performances under fermentative and stressing conditions

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Abstract

Aims: The aim of this research was to study the effect of time, temperature, sugar content and addition of diammonium phosphate (DAP) on ochratoxin A (OTA) removal by two strains of *Saccharomyces cerevisiae* using a completely randomized design.

Methods and Results: The strains were grown in a medium containing OTA (2 μg l^{-1}), two sugar levels (200 and 250 g l^{-1}), with or without DAP (300 mg l^{-1}), and incubated at 25–30°C. The yeasts were able to decrease the toxin amount by *c.* 70%, with the highest removing effect observed after 3 days at 30°C in the presence of 250 g l^{-1} of sugars and with DAP; after 10 days, the toxin was partially released into the medium. The strains produced high ethanol and glycerol contents, showed high tolerance to single/combined stress conditions and possessed *β*-D-glucosidase, pectinase and xylanase activities.

Conclusions: Ochratoxin A removal was affected by time, temperature, sugar and addition of DAP. Moreover, the phenomenon was reversible.

Significance and Impact of the Study: Ochratoxin A removal could be an interesting trait for the selection of promising strains; however, the strains removing efficiently the toxin could release it back; thus, the selection of the starter should take into account both the removal and the binding ability of OTA.

Introduction

The key role of yeasts in conducting the alcoholic fermentation has long been recognized (Fleet 1999; Mortimer and Polsinelli 1999), and many factors have been found to influence their growth and metabolism and, as a consequence, the quality of wine (Charoenchai *et al.* 1998; Torija *et al.* 2003; Mendes-Ferreira *et al.* 2004). The temperature is a significant parameter for the course of wine fermentation (Pizarro *et al.* 2008), as it can strongly impact on yeast growth (Charoenchai *et al.* 1998), sugar utilization and ethanol production (Torija *et al.* 2003). Another significant variable is the concentration of fermentable sugars in grape musts (Charoenchai *et al.* 1998); concentrations from 200 to 300 g l^{-1} hinder yeast growth and decrease both the maximum population and ethanol yield (D'Amato *et al.* 2006). Furthermore, nitrogen can often be a limiting factor for yeast growth and sugar attenuation (Mendes-Ferreira *et al.* 2004). A value <150 mg l^{-1} of yeast assimilable nitrogen (YAN) in the must is associated with a higher probability of fermentation problems such as sluggish or stuck fermentations (Henschke and Jiranek 1993). In these cases, the addition of diammonium phosphate (DAP) or, to a lesser extent, organic yeast nutrients to nitrogen-deficient musts is a widely used practice (Barbosa *et al.* 2012).

Ochratoxin A (OTA) is a potent nephrotoxic and hepatocarcinogenic mycotoxin, recognized as a possible human carcinogen by the International Agency for Research on Cancer (Cecchini *et al.* 2006). The presence of the toxin in musts and wines is due to fungal contamination of the grapes by *Aspergillus* and *Penicillium* genera that may develop in both pre- and postharvest or during the phases before the winemaking (Esti *et al.* 2012).

"People are often unreasonable, irrational, and self-centered.
Forgive them anyway.
If you are kind, people may accuse you of selfish, ulterior motives.
Be kind anyway.
If you are successful, you will win some unfaithful friends and some genuine enemies.
Succeed anyway.
If you are honest and sincere people may deceive you.
Be honest and sincere anyway.
What you spend years creating, others could destroy overnight.
Create anyway.
If you find serenity and happiness, some may be jealous.
Be happy anyway.
The good you do today, will often be forgotten.
Do good anyway.
Give the best you have, and it will never be enough.
Give your best anyway."

Blessed Teresa of Calcutta (1910-1997)