UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Etjen BIZAJ

INTERACTIONS BETWEEN CONTAMINANTS AND STARTER CULTURES DURING ALCOHOLIC FERMENTATION

DOCTORAL DISSERTATION

Ljubljana, 2013

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MEDSEBOJNI VPLIV KONTAMINANTOV IN STARTERSKIH KULTUR MED ALKOHOLNO FERMENTACIJO

DOKTORSKA DISERTACIJA

Ljubljana, 2013

On the basis of the Statute of the University of Ljubljana and by decisions of the Senate of the Biotechnical Faculty and the decision of University Senate, dated from September 28th 2009, the continuation to doctoral Postgraduate Study of Biological and Biotechnical Sciences, field: Biotechnology, was approved. Prof. Peter Raspor, PhD, as the supervisor, and Chris Curtin, PhD, as the co-advisor, were confirmed.
Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Senata Univerze z dne 28.09.2009 je bilo potrjeno, da kandidat izpolnjuje pogoje za neposreden prehod na doktorski Podiplomski študij bioloških in biotehniških znanosti ter opravljanje doktorata znanosti s področja biotehnologije. Za mentorja je bil imenovan prof. dr. Peter Raspor ter za somentorja dr. Chris Curtin.

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The dissertation is a result of my own research. I declare that all scientific articles in the dissertation are identical to the published version.

Etjen Bizaj

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AI Overall quality of food is determined by its safety and by its sensory properties. The latter is of particular importance in wine production, since it is the most important advantage for the consumers, however its safety has to be put at the base of all processes involved. Alcoholic fermentation which is lead by wine yeasts is the most crucial process during wine production and the above mentioned two reasons lead us to the aim of this thesis; to improve the potential of industrial wine yeasts for flavor production during alcoholic fermentation and elucidate their sensitiveness to contaminants (phytopharmaceuticals and mycotoxins) as well as to determine their potential to remove these from grape juice/ wine and to improve its safety. A novel strategy of the combination of classical breeding methods was successfully used, to hybridize industrial flavor-active alloploids (*S. cerevisiae* x *S. kudriavzevii*) with an industrial mutant of *S. cerevisiae* with low H_2S phenotype, harnessing for the first time laboratory produced food-grade triple hybrids expressing good fermentation performance, high concentrations of flavor active compounds and low H_2S phenotype, this way confirming hypothesis (A). Interactions of three types of complementary contaminants during alcoholic fermentations; on one hand phytopharmaceuticals (pyrimethanil and fenhexamid) used for treatments against molds which produce mycotoxins, and on the other ochratoxin A produced by molds on grapes were analyzed with genetically different wine yeast strains in stationary and fermentative assays. During stationary and fermentative interactions in synthetic media as well as in the fermentative interactions with contaminants in grape juice the contaminants are removed by adsorption and not metabolic action by the yeast strains. The removal potentials of the yeast strains in stationary and fermentative stages are different. Yeast strain and chemical contaminant related removal dependency was observed as well. Hypothesis (B), (C) and (D) were confirmed. On the other hand, all three contaminants were able to impair on the fermentation kinetics and exo-metabolome of yeast strains in fermentative assays. Strain and chemical contaminant dependency were observed in this type of interaction. Hypothesis (E) and (F) were confirmed. Finally, all the contaminants present in the media negatively affected the alcoholic fermentation performance of all the genetically different wine yeasts, as well as their exo-metabolome. Even though they are complimentary, their concentration should be at the lowest possible level in grape juice.

KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD Dd
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KG vino/ kvasovke / hibridi / *Saccharomyces sensu stricto* / interakcije / starter kulture / klasične plemenilne metode / kontaminanti / pirimetanil / fenheksamid / ohratoksin A / alkoholna fermentacija / fermentacijski produkti / zmanjšanje koncentracije kontaminantov
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SA RASPOR, Peter (mentor) / CURTIN, Chris (somentor)
KZ SI-1000 Ljubljana, Jamnikarjeva 101
ZA Univerza v Ljubljani, Biotehniška fakulteta, Podiplomski študij bioloških in biotehniških znanosti, področje biotehnologije
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TD Doktorska disertacija
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AI Na splošno je kakovost hrane definirana kot vsota vseh njenih dobrih in slabih lastnosti, od teh pa sta izjemnega pomena njena varnost in senzorične značilnosti. Prva mora biti osnova vsakega proizvodnega procesa, druga pa je njena najpomembnejša dodana vrednost, ki je tudi pri vinu zelo pomembna. Alkoholna fermentacija grozdnega soka je najbolj kritična faza pridelave vina in prej omenjeni lastnosti sta bili povod za to raziskavo; njen temeljni cilj je bil izboljšati potencial industrijskih vinskih kvasovk za proizvodnjo zaželenih hlapnih spojin med alkoholno fermentacijo in razkriti njihovo občutljivost na kontaminante (fitofarmacevtska sredstva in mikotoksine), kot tudi določiti potencial industrijskih sevov za odstranjevanje le-teh iz grozdnega soka/vina in s tem povečati varnost. Razvita je bila nova strategija kombinacije klasičnih plemenilnih metod, s katero sta bila prvič doslej križana aloploid (*S.cerevisiae* x *S.kudriavzevii*) z visokim potencialom za produkcijo zaželenih hlapnih snovi ter mutant (*S.cerevisiae*) s fenotipom nizke proizvodnje H_2S. S tem so bili prvič doslej laboratorijsko proizvedeni industrijski trojni hibridi z dobrim fermentacijskim potencialom ter visokim potencialom za proizvodnjo zaželenih in nizkim potencialom za proizvodnjo nezaželenih hlapnih spojin. Potrjena je bila hipoteza (A). Mesebojni vplivi sevov genetsko različnih vinskih kvasovk so bili analizirani s tremi komplementarnimi kontaminanti; fitofarmacevtskima sredstvoma (pirimetanil in fenheksamid) v splošni rabi za tretiranje proti plesnim na grozdju, ki proizvajajo mikotoksine, in ohratoksinom A. Analize medsebojnih vplivov so bile izvedene tako v stacionarnih kot fermentativnih pogojih, v sintetičnem gojišču in grozdnem soku. V vseh primerih se izkaže, da so vsi kvasni sevi sposobni zmanjšati koncentracijo kontaminantov, a le z adsorpcijo in ne metabolno razgradnjo. Potencial kvasne biomase med stacionarnim in fermentativnim procesom se razlikuje; opazna pa je tudi sevna in kontaminantna odvisnost. S tem so bile potrjene hipoteze (B), (C) in (D). Vsi trije kontaminanti so negativno vplivali na fermentacijsko kinetiko in potencial za proizvodnjo zaželenih hlapnih snovi; tudi v tem primeru se je pokazala sevna in kontaminantna odvisnost medsebojnih vplivov. Hipotezi (E) in (F) sta bili potrjeni. Delo kaže, da ne glede na komplementarnost vsi trije kontaminanti negativno vplivajo na kakovost vina, proizvedenega z genetsko različnimi vinskimi kvasnimi sevi. Koncentracija le-teh naj bo v grozdnem soku čim manjša, saj to omogoča boljšo kakovost vina.

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Bizaj E., Čuš F., Raspor P. 2011. Removal of pyrimethanil and fenhexamid in *Saccharomyces cerevisiae* liquid cultures. Food Technology and Biotechnology, 49, 4: 474-480

Bizaj E., Cordente A.G., Bellon J.R., Raspor P., Pretorius I.S. 2012. A breeding strategy to harness flavour diversity of *Saccharomyces* spp. interspecific hybrids and minimize hydrogen sulfide production. FEMS Yeast Research, 12, 4: 456-465

Other unpublished scientific articles

Bizaj E., Curtin C., Čadež N., Raspor P. 2012. Interactions between industrial yeasts and contaminants in grape juice affect wine composition profile. Journal of Agricultural and Food Chemistry, Submited for peer review.

1 INTRODUCTION

1.1 OVERVIEW OF RESEARCH FIELD

Fermentation, the microbial degradation of organic compounds without net oxidation, is an important process in the global carbon cycle and is exploited worldwide for food-processing technologies (Sieuwerts et al., 2008).
The link between food and microbiology was laid by Pasteur, who found that yeasts were responsible for alcoholic fermentation (Mortimer, 2000), and alcoholic fermentation of grape juice into wine, is amongst the most important global processes in food technology and economics (Pretorius, 2000).
The alcoholic fermentation of grape juice, when sugars are transformed into alcohol is conducted by yeasts; their ecology and physiology is influenced by many factors: varietal, environmental, agronomic, and technological (Pretorius, 2000; Garca et al., 2004; Ribereau-Gayon et al., 2006).
Interactions between microbes and chemical compounds in grape juice occur via multiple mechanisms. The effects of such interactions on the fitness of microbes involved, may either be positive, neutral, or negative (Sieuwerts et al., 2008).
Overall quality of food and therefore wine as well, is defined as the sum of all positive and negative features that a product has. Food safety is the base for the quality of wine. Nowadays consumers are very careful and informed about healthy diets and that is why novel technologies are being developed in the wine processing industry to improve its safety.
Grapes from which wine is produced are coming from vineyards, where during the growing season harsh weather conditions may occur, due to which the crop may be under threat. This would cause lower quality grape and consequently wine (Ribereau-Gayon et al., 2006).
Grapes may be threatened by microbiological spoilage with molds, bacteria and yeasts that cause chemical composition changes by transforming compounds already present into unwanted ones such as ethanol, acetic acid, glucuronic acid, etc., and producing completely new toxic ones by secondary metabolism, such as mycotoxins (Calonnec et al., 2004; Bellí et al., 2006; Ribereau-Gayon et al., 2006). On the other hand, the use of pesticides in order to preserve the crop from microbial and other spoilage induces changes in grape and later grape juice chemical composition as well (Cabras and Angioni, 2000).

In recent times due to the toxicity of phytopharmaceuticals, their use has been drastically lowered during the grape growing season, this way lowering their concentration in the final product (Cabras and Angioni, 2000). On the other hand by lowering the intensity of treatments, it has been demonstrated that the unwanted and toxic compounds produced by spoilage microorganisms rise, this way lowering the product's safety and threatening human's health (Lo Curto et al., 2004; Pardo et al., 2005; Ribereau-Gayon et al., 2006).
Treatments during the grape growing season are inevitable and in difficult years, when the conditions for infection by the spoilage microorganisms are extremely high, treatments are sometimes being overused. However, even though the treatments are applied, due to technical and varietal differences, the presence of infected grapes is almost inevitable as well as the presence of unwanted compounds (Cabras and Angioni, 2000; Ribereau-Gayon et al., 2006).

In wine production *Botrytis cinerea* is still considered the most dangerous threat (Rosslenbroich and Stuebler, 2000). Not only its direct effect by infecting grapes and negatively changing their composition, its infection favors the infection of other dangerous

spoilage microorganisms such as lactic acid and acetic acid bacteria. Molds of genera *Aspergillus* and *Penicillium*, especially *A.carbonarius* are considered the most important producers of mycotoxins; ochratoxin A, they reduce wine's safety and consequently its quality (Lo Curto et al., 2004; Ribereau-Gayon et al., 2006; Mateo et al., 2007).
Ochratoxin A is a strong nephrotoxic, carcinogenic, immunotoxic and teratogenic mycotoxin that can contaminate various foods and beverages (Mateo et al., 2007). Its presence in grapes (< 3–311 ng/L) and wines (< 3–388 ng/L) was reported for the first time in 1996 (Zimmerli and Dick, 1996). Some studies suggest that wine is the second major source of OTA intake after cereals (Mateo et al., 2007). OTA concentration in wines is dependent upon climate, being higher in regions with higher temperatures, and also the type of wine. Higher concentrations were found in dessert wines, and red still dry wines in comparison to white and sparkling (Zimmerli and Dick, 1996; Mateo et al., 2007). However the European Union has established a maximum allowable concentration of OTA in wines from the vintage 2005 of 2ppb (EC Regulation, 2005).

During the growing season, many types of pesticides are being used; insecticides, herbicides and acaricides, however the most used are fungicides. The latest, because of *Botrytis* (botriticides) are the most present later in grape juices and consequently wines (Cabras and Angioni, 2000; Leroux, 2000; Dugan et al., 2002).
Because of its adaptability and the various strains of *Botrytis cinerea* that are present in nature, there is a very high possibility of microorganisms becoming resistant to botriticides; that is why new types of active components and adjuvants are being constantly developed (Elad and Stewart, 2004).
One of the most widely used botryticides of the older generation is pyrimethanil. Pyrimethanil (*N*-(4,6 dimethylpyrimidin-2-yl)-aniline) which is a colorless, crystalline substance, is practically insoluble in water and belongs to the anilinopyrimidine class (Tomlin, 1994). With a lesser ecological impact, one of the new types is fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide), which belongs to the chemical class of hydroxyanilides (Tomlin, 1994). This fungicide is less eco-toxic because it is readily degraded into non-toxic derivatives (Anderson, 1999; Leroux et al., 1999; Leroux, 2000).

To improve the safety of wine, during wine production it has been found that the concentration of contaminants can be lowered by different types of processing (Miller et al., 1985; Tsiropoulos et al., 2005; Kaushik et al., 2008; Čuš et al., 2010). Indeed, 50-80% of contaminants were found to be removed from the concentration early present in crushed grapes because they are bond to the discarded skins and seeds (Leong et al., 2006). During different types of clarification before fermentation with bentonite and gelatine a moderate removal of contaminants, has also been found (Cabras et al., 1997; Cabras et al., 1998; Cabras et al., 1999; Tsiropoulos et al., 1999).
The ability of yeasts to decrease pesticide residues in enological conditions is well known. Studies concerning a large number of classes of pesticides showed that yeasts can decrease the amount of pesticides by degradation and/or adsorption (Pérez-Serradilla and Luque de Castro, 2008; Pinna et al., 2008), however the latter was found to be the most effective and frequent (Cabras and Angioni, 2000). Moreover, a combined action of adsorption/degradation has been reported as well (Cabras et al., 2000).
Yeasts, yeast cell walls or yeast cell wall extracts were either found to be able to remove OTA in synthetic media as well as in wine processing (Gambuti et al., 2005; Ratola et al., 2005; Caridi, 2007; Fernandes et al., 2007; Hocking et al., 2007). OTA is thought not to be degraded (biologically transformed) by yeasts during primary alcoholic fermentation; rather, the toxin is

adsorbed. However, there is no firm consensus about the proposed mechanism involved in the removal of OTA (Bejaoui et al., 2004; Caridi et al., 2006; Cecchini et al., 2006; Hocking et al., 2007).
The main agent for adsorption of contaminants is the yeast cell wall, which contains polysaccharides as basic building blocks. Therefore, it offers host functional groups capable of xenobiotic binding (Ballou, 1988). A great diversity has been observed among yeasts for their adsorption activity: the outermost layer of the cell wall has a chemical composition that varies notably from yeast to yeast, and adsorption is very strain dependent. Moreover, media composition and physico-chemical conditions were shown to strongly affect the removal potential of yeast strains (Huwig et al., 2001; Caridi, 2006; Caridi, 2007).
Nunez and colleagues in 2007 demonstrated that the main fraction of mannoproteins is released in the first week after the completion of alcoholic fermentation and that during this stage the predominant adsorptive action is observed; in this way determining the total removal potential of the yeast (Nunez et al., 2007). This suggests that by using the technology of »battonage«; that is an extended yeast lees contact (Ribereau-Gayon et al., 2006), after the end of alcoholic fermentation, an additional amount of contaminants can be removed from wine improving its quality.

The most important added value and a component of quality besides safety is the aromatic composition of wines. Wine's aromatic profile is composed by primary aroma (varietal and pre-fermentative), which comes from the grape must; secondary or fermentative aroma, a group of volatile substances that appear during fermentation, and tertiary or post-fermentative aroma, which develops during aging and conservation of wines (Ribereau-Gayon et al., 2006). Alcoholic fermentations of grape juices are conducted by wine yeasts. During alcoholic fermentation the aromatic profile of the media drastically changes, which means that wine yeasts are strongly determining the wine's aromatic profile (Pretorius, 2000; Fleet, 2003).
In recent years the trend has been to use selected yeasts for the alcoholic fermentation, because these can guarantee a smooth development of the process, avoid the production of off-flavors and the generation of positive aromas for the sensory properties of wine (Pretorius and Bauer, 2002). In order to improve the wine's aromatic composition other alternative techniques of inoculation have been adopted; mixed/sequenced inocula of different strains (Ciani et al., 2006; King et al., 2010), introduction of non-*Saccharomyces* selected yeasts (Jolly et al., 2003; Jolly et al., 2006) and the use of interspecies hybrids of *Saccharomyces sensu stricto* yeasts (Swiegers et al., 2009; Bellon et al., 2011).
The improvement of wine aromatic profiles is therefore strongly dependent on the improvement of starter cultures, meaning wine yeasts. Strain improvement strategies are numerous − often complementary to each other, and the choice among them is based on three factors: (i) the genetic nature of traits (monogenic or polygenic); (ii) the knowledge of the genes involved (rational or blind approaches); and (iii) the phenotypic trait requirement (Giudici et al., 2005). Due to lack of acceptance for the use of recombinant yeasts, only classical techniques such as clonal selection of variants, mutation and selection, and mating/hybridization are currently being used to produce food-grade starter cultures (Pretorius, 2000; Schilter and Constable, 2002).
Improvement of wine yeast properties means enhancing fermentation performance, as well as optimizing the production of yeast secondary metabolites such as volatile aroma compounds.
Some compounds are detrimental to wine quality and therefore their production should be minimized. A well studied example is hydrogen sulfide (H_2S); a yeast metabolite that imparts a rotten egg off-flavour in wine and has a very low sensory threshold; between 50-80 µg/L (Rauhut, 1993). During alcoholic fermentation, H_2S is produced by yeasts, and is strain

dependent (Mendes-Ferreira et al., 2002; Nowak et al., 2004). However, not all *S. cerevisiae* are able to produce H_2S; 1% of the naturally occurring population is unable to produce this compound (Zambonelli et al., 1984).
The majority of H_2S produced during winemaking occurs as a result of the biosynthesis of the sulphur containing amino acids methionine and cysteine, which occur in low concentrations in grape juice, through the *sulfate reduction sequence* (SRS) (Takahashi et al., 1980; Stratford and Rose, 1985).
Several genetic engineering strategies have been used for limiting H_2S production by yeast, which involved the inactivation or over expression of genes involved in the SRS (Tezuka et al., 1992; Omura et al., 1995; Sutherland et al., 2003). However, only in the work of Cordente et al., (2009), a food-grade set of low H_2S producing mutants of a commercial wine yeast was developed. Specific mutations in the *MET10* and *MET5* genes, which encode the two catalytic subunits of the sulfite reductase enzyme, the key enzyme in the SRS pathway, were found to be responsible for the low H_2S producing phenotype of these strains. Although the inactivation, of yeast sulfite reductase has been shown to dramatically decrease H_2S production, it also leads to an increase in SO_2 production in brewing and enological conditions (Hansen and Kielland-Brandt, 1996; Cordente et al., 2009), which can interfere with the biological deacidification in wine, due to the well known negative effect of SO_2 on malolactic bacteria (Versari et al., 1999).
The enhancement of formation of positive aroma compounds by wine yeasts during alcoholic fermentation, belonging to chemical families such as aldehydes, ketones, alcohols, acids, and esters by wine yeasts has also been a priority (Pretorius, 2000; Pretorius and Bauer, 2002; Fleet, 2003).
A recent approach to the improvement of wine yeast strains, involved the generation of interspecies hybrids within the *Saccharomyces sensu stricto* group (Bellon et al., 2011). The out-coming novel hybrids, show intense positive flavor production characteristics; which have also been previously highlighted (Swiegers et al., 2009).
Natural interspecies hybrids were previously isolated from wine fermentation (Gonzalez et al., 2006), while the lager yeast *S. pastorianus* is in fact a natural hybrid of *S. cerevisiae and S. bayanus* (Sipiczki, 2008). Moreover, through application of molecular identification methods, natural double (Sipiczki, 2008) and triple hybrids (*S. cerevisiae* x *S. uvarum* x *S. kudriavzevii*) (Masneuf et al., 1998; de Barros Lopes et al., 2002; Gonzalez et al., 2006) have been identified as well.
The potential of interspecies hybrids to be involved in further improvement by classical breeding methods have always been underestimated. Generally considered sterile and a reproductive 'dead-end', *Saccharomyces* interspecies hybrids can potentially undergo further hybridization (de Barros Lopes et al., 2002).
The combination of classical breeding methods should be leading into novel food-grade industrial strains with combined and intensively expressed positive enological traits, as well as minimizing the negative ones.

The effect of microbial presence and/or action during alcoholic fermentation on the concentration of contaminants has been widely studied. On the other hand, the research focus on the effect of contaminants on the fitness as well as oenological traits of wine yeasts has not been very intensive.
Pesticides were found to affect wine yeasts under certain conditions. Pyrimethanil was found to significantly diminish the anaerobic growth of *Hanseniaspora uvarum* in synthetic media (Čus and Raspor, 2008), and in some cases the presence of pesticides have been found to stimulate yeasts, particularly *Kloeckera apiculata*, which produced higher concentrations of

alcohol (Cabras et al., 1999). Fenhexamid, for example, did not affect the alcoholic fermentation performance of wine yeasts (Cabras et al., 2003).
The presence of fungicides during alcoholic fermentation was found to affect yeast's exo-metabolome. Various pesticides have been shown to affect the aromatic profile of red and white grape vine varieties produced by starter cultures of different genetic backgrounds; not including interspecies hybrids (Oliva et al., 1999; Garcia et al., 2004; Oliva et al., 2008; González-Rodríguez et al., 2011; Noguerol-Pato et al., 2011).
If on one hand the effect of pesticides on wine yeasts during alcoholic fermentation is poorly researched, there is no present data about ochratoxin A's possible effect.

1.2 THE RESEARCH AIM AND HYPOTHESIS

The aim of our research was to elucidate how the overall quality of wine; that is its safety and its sensory characteristics are being affected by interactions that occur during alcoholic fermentation, in-between genetically different wine yeasts and contaminants commonly present in grape juices before alcoholic fermentation. The development of novel *Saccharomyces sensu stricto* interspecies hybrids, for the purpose of improvement of wine quality and stabilization of production technology has been performed as well.
The purpose was to develop novel food grade industrial strains with improved enological traits such as; fermentation performance, improvement of the production of desirable flavors and reduction of the production of off-flavors, improved removal of contaminants from wine and resistance to their effects for the improvement of the wine's overall quality.
In regard to what is mentioned, the following work hypotheses were chosen:

A) Novel *Sacharomyces sensu stricto* interspecies hybrids show positive parental inheritance.

B) Yeast biomass is able to remove the contaminants from media.

C) The removal capacity of metabolically active biomass during alcoholic fermentation is different from the removal capacity of metabolically active biomass after the end of alcoholic fermentation.

D) The removal capacity is dependent upon strain and contaminant type.

E) High concentrations of ochratoxin A and phytopharmaceuticals in the media affect the fermentation kinetics; the affection of kinetics is strain and contaminant type dependent.

F) High concentrations of ochratoxin A and phytopharmaceuticals in the media affect the yeast metabolome during alcoholic fermentation; the affection is strain and contaminant type dependent.

2 SIENTIFIC PUBLICATIONS

2.1 PUBLISHED ARTICLES

2.1.1 Removal of ochratoxin A in *Saccharomyces cerevisiae* liquid cultures

Odstranjevanje ohratoxina A v tekočih medijih s *Saccharomyces cerevisiae*

Etjen Bizaj, Jan Mavri, Franc Čuš and Peter Raspor

South African Journal of Enology & Viticulture (2009), 30, 2: 151-155

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

Removal of Ochratoxin A in *Saccharomyces cerevisiae* Liquid Cultures

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Yeast strains

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

Cultivation and medium

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

Culture conditions and sampling

The inocula were prepared as described above and added directly to the fermentors, to give a final concentration of $1*10^6$ CFU/mL in the medium. The kinetics of fermentation were followed by CO_2 release measurement (weight loss). The fermentations were carried out at 20°C. The samplings for determination of the OTA concentration in the media were done at three stages: at time 0, at the end of the fermentation (less than 2 g/L of resting reducing sugars) and after seven days of contact with yeast biomass. After seven days of biomass-media contact, yeast biomass was also collected for OTA determination in the biomass and the fermented media. The latter were also analysed for volatile acidity and concentration of reducing sugars. Biomass was collected by centrifugation; three times at 11 200 x g for 10 min to remove supernatant.

Biomass recovery for stationary test

For the stationary test, the biomass produced during fermentations under anaerobic conditions (Čuš & Raspor, 2008) in liquid YM, with 18.0% of glucose at pH 6.37, was recovered by centrifugation (10 min, 11 200 x g) and washed three times (10 min, 11 200 x g) with phosphate buffer (pH 6.37, sterilised by membrane filtration (0. 20 µm, Sartorius, Germany). Finally, the cells were resuspended in the phosphate buffer to give a final concentration of 0.44 g/mL.

Stationary tests for biomass reduction of ochratoxin A

The test was performed in two replicates in tubes containing 5 mL of assay solution, consisting of 4.890 mL of phosphate buffer (pH 6.37), with 0.010 mL of stock solution of OTA (Sigma-Aldrich, USA) added to give a final concentration of 5.00 ng/mL at 20°C. To each tube, 0.1 ml of a yeast biomass suspension (viable or non-viable cells) in phosphate buffer was added to give a final concentration of 0.044 g/mL. Non-viable cells were prepared by the addition of Na-azide (Sigma-Aldrich, USA) to biomass suspended in phosphate buffer (0.025% final concentration in media) in order to exclude metabolic action and preserve the structural integrity of the cells (Bejaoui *et al*., 2004; Nunez *et al*., 2007). After seven days of contact between the biomass and the media (Martínez-Rodriguez *et al*., 2001a,b; 2002; Nunez *et al*., 2007), with or without agitation (rotary shaking of tubes at 190 min^{-1}), the solution was centrifuged and the non-adsorbed amounts of OTA in the medium were measured.

TABLE 1

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

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

Start fermentation T = 0 days.
End fermentation: < 2 g/L glucose.
7 days after fermentation (daily mixing).
a,b,c : significant difference at $P \leq 0.05$.

Extraction of OTA from yeast biomass and YM media

The extraction of OTA from yeast biomass and YM samples was performed using the organic extraction protocol for wine and grape samples, proposed by the manufacturer of the enzyme immunoassay kit used, I'screen Ochra (Tecna S.r.l., Trieste, Italy), which in our case was modified for 500 µL sample volumes. Briefly, 100 mg of sample wet biomass was first transferred in a 2 mL Eppendorf tube, washed two times with 1 mL of distilled water, centrifuged (5 min, 1 500 g) and resuspended in 400 µL of H_2O. In the case of YM media, 500 µL of sample was collected in the tube without further treatment. To each sample, 500 µL 1M HCl and 1 mL of dichloromethane were added and mixed at 1 200 rpm in a rotary shaker (TTS2, IKA, USA) for 15 min at room temperature. This was followed by centrifugation at 2 200 g for 15 min. Then, 500 µL of the lower dichlormethane phase was pipetted in another 2 mL Eppendorf tube and 250 µL of 130 mM $NaHCO_3$ (pH 8.1) was added. This was mixed again for 15 min at 1 200 rpm. Finally, samples were centrifuged at 2 200 g for 15 min, and 200 µL of the upper polar phase was taken and analysed with the immunoassay kit.

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

Determination of OTA residues

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

Determination of reducing sugars and volatile acidity

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

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

Statistical analysis

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

RESULTS AND DISCUSSION

OTA removal during alcoholic fermentation and yeast biomass contact

Fermentation kinetics (Fig. 1), which were followed by the daily release of CO_2 (weight loss), showed no significant differences between the controls and the fermentations with added OTA. After the completion of fermentation, the yeast biomass was maintained in contact with the media for seven days by daily mixing. During this time, the major proportion of mannose was released from the yeast cell walls into the media (Nunez *et al*., 2007) due to accelerated autolysis triggered by the lack of nutrients (Martinez-Rodriguez *et al*., 2001a; 2001b; 2002). However, during the alcoholic fermentation conducted by both yeast strains, no significant reduction was found in the concentration of OTA. The concentrations detected were 3.41 ng/mL and 4.82 ng/mL, for ZIM 1927 and Lalvin EC-1118 respectively (Table 1). On the other hand, a significant reduction was observed after seven days of contact between the media and yeast biomass. The final concentrations detected in the media were 2.46 ng/mL and 3.69 ng/mL for ZIM 1927 and Lalvin EC-1118 respectively. Under the conditions used in our study, the two strains were unable to significantly reduce the concentration of OTA in synthetic media during the alcoholic fermentation phase. This result contrasts with that reported by Bejaui *et al*. (2004), who used different conditions that have been found to affect OTA removal potential: pH, media and strains (Bejaoui *et al*., 2004; Caridi, 2006; 2007; Cecchini *et al*., 2006). In the post-fermentation period, 25.4% (Lalvin EC-1118) and 29.3% (ZIM 1927) reductions were achieved by the yeast strains. At the same time, although the initial concentration of OTA in the media was different for the two strains, the degree of removal induced by the two strains was not significantly different. These results suggest that the release of mannoproteins from cell walls after the completion of fermentation (Caridi, 2006; 2007; Nunez *et al*., 2007) is important in the removal of OTA.

Concentration of OTA in wet yeast cell biomass

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

OTA removal in yeast stationary phase biomass tests

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

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

statistically significantly different compared to the trials with the strain ZIM 1927 viable and non-viable biomass when agitation was not applied, when only 21.0 and 21.4% removal respectively was obtained. The trend of a higher degree of OTA removal in agitated assays when compared to non-agitated assays of the same strain and viability is strongly present (Table 2). However, this is confirmed statistically only in ZIM1927 non-viable biomass assays. For strain Lalvin-1118-EC there were not statistically significant differences between the viable and non-viable yeast biomasses. Along with the confirmation that the mechanism of OTA removal by these two *S. cerevisiae* yeast strains involves adsorption, the extent of adsorption is perhaps lower than it might be at a lower pH (Bejaoui *et al*., 2004; Ringot *et al*., 2005). Adsorption is affected by pH because of different adsorption mechanisms used by different microorganisms, and because of cell wall composition (Huwig *et al*., 2001).

Volatile acidity and reducing sugars

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

TABLE 2

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

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

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

TABLE 3

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

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

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

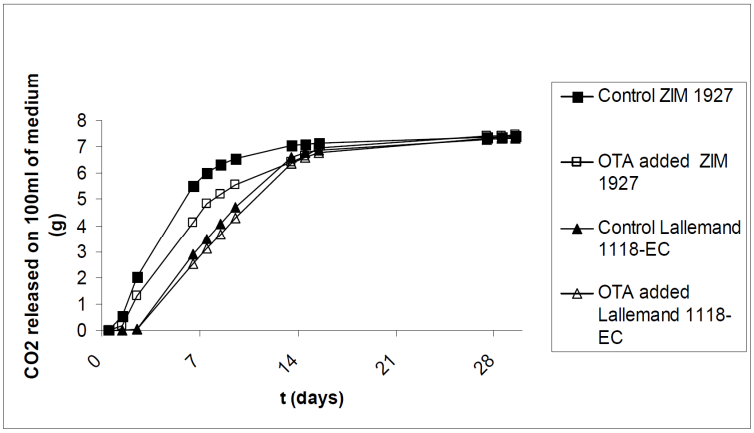

FIGURE 1

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

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

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

CONCLUSIONS

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

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

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

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2.1.2 Removal of pyrimethanil and fenhexamid in *Saccharomyces cerevisiae* liquid cultures

Odstranjevanje pirimetanila in fenhexamida v tekočih medijih s *Saccharomyces cerevisiae*

Etjen Bizaj, Franc Čuš and Peter Raspor

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The capacity for the removal of pyrimethanil and fenhexamid, two fungicides commonly used for the control of *Botrytis cinerea* in vineyards, has been evaluated during an alcoholic fermentation process in batch system. Commercial and wild strains of *Saccharomyces cerevisiae* were used. Batch fermentations were carried out in yeast extract-malt extract medium (YM) with 18.0 % (by mass) glucose, and the fungicides were added separately at three concentrations: 0.1, 1.0 and 10.0 mg/L. The removal capacity of yeast strains was also examined in stationary phase cultures of *Saccharomyces cerevisiae*. Stationary assays were performed with yeast biomass harvested from the stationary phase of an anaerobic fermentation process, with separate additions of 0.1, 1.0 and 10.0 mg/L of both fungicides. Removal studies with stationary phase cells were performed with viable and non-viable cells inactivated with sodium azide. This study clearly shows that both *Saccharomyces cerevisiae* strains were able to remove fenhexamid and pyrimethanil in stationary and fermentative assays. The removal potential is shown to be strain dependent in stationary but not in fermentative assays. However, the removal potential is dependent on the type of fungicide in both stationary and fermentative assays. In stationary phase cultures no significant difference in fungicide removal potential between viable and non-viable cells was observed, indicating that both pesticides were not degraded by metabolically active cells. However, the presence of both pesticides influenced fermentation kinetics and only pyrimethanil at 10.0 mg/L increased the production of volatile acidity of both strains.

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Removal of Pyrimethanil and Fenhexamid from *Saccharomyces cerevisiae* Liquid Cultures

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Summary

The capacity for the removal of pyrimethanil and fenhexamid, two fungicides commonly used for the control of *Botrytis cinerea* in vineyards, has been evaluated during an alcoholic fermentation process in batch system. Commercial and wild strains of *Saccharomyces cerevisiae* were used. Batch fermentations were carried out in yeast extract-malt extract medium (YM) with 18.0 % (by mass) glucose, and the fungicides were added separately at three concentrations: 0.1, 1.0 and 10.0 mg/L. The removal capacity of yeast strains was also examined in stationary phase cultures of *Saccharomyces cerevisiae*. Stationary assays were performed with yeast biomass harvested from the stationary phase of an anaerobic fermentation process, with separate additions of 0.1, 1.0 and 10.0 mg/L of both fungicides. Removal studies with stationary phase cells were performed with viable and non-viable cells inactivated with sodium azide. This study clearly shows that both *Saccharomyces cerevisiae* strains were able to remove fenhexamid and pyrimethanil in stationary and fermentative assays. The removal potential is shown to be strain dependent in stationary but not in fermentative assays. However, the removal potential is dependent on the type of fungicide in both stationary and fermentative assays. In stationary phase cultures no significant difference in fungicide removal potential between viable and non-viable cells was observed, indicating that both pesticides were not degraded by metabolically active cells. However, the presence of both pesticides influenced fermentation kinetics and only pyrimethanil at 10.0 mg/L increased the production of volatile acidity of both strains.

Key words: alcoholic fermentation, *S. cerevisiae*, pyrimethanil, fenhexamid, synthetic media

Introduction

Pesticides have proven beneficial effects on the preservation of crop yield. However, they are extensively used, and sometimes overused, posing serious human health concerns (*1*). In wine production *Botrytis cinerea* is a fungal pathogen of serious economic importance. Because of its increasing tolerance to the old generation of fungicides, the treatments have become more severe, and consequently new and more effective pesticides are being developed. These are characterized as being less toxic for human health and have a lesser ecological impact (*2–4*).

One of the most widely used botryticides is pyrimethanil. Pyrimethanil (*N*-(4,6-dimethylpyrimidin-2-yl)-aniline) is a colourless, crystalline substance, which is practically insoluble in water and belongs to the anilinopyrimidine class (*5*). With a lesser ecological impact, one of the new types is fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide), which belongs to the chemical class of hydroxyanilides (*6*). This fungicide is less ecotoxic because it is readily degraded to non-toxic derivatives (*7–9*).

Stronger concentrations are used during the years when the conditions for *Botrytis* infection are extremely good, which can lead to the presence of pesticide resi-

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dues on grapes; sometimes higher than their maximum permitted levels (*2*).

Even though the solubility and stability of a pesticide can influence the concentrations of residues on grapes, residue levels are also influenced by the type of handling and processing (*10*,*11*). The fact that all wines have been found to have lower concentrations of residual pesticides than detected on the corresponding grapes confirms this observation (*12–17*).

Yeasts, during alcoholic fermentation, as well as other types of processing, have the ability to decrease pesticide residues. Studies concerning a large number of classes of pesticides showed that yeasts can decrease the amount of pesticides by degradation and/or adsorption, the latter of which was found to be the most effective and frequent (*1*). The removal of toxic pesticides during wine processing has been widely studied (*18*,*19*).

The main agent for adsorption is the yeast cell wall, which contains polysaccharides as basic building blocks. Therefore, it offers host functional groups capable of xenobiotic binding (*20*). Nuñez *et al.* (*21*) demonstrated that the main fraction of mannoproteins is released in the first week after the completion of alcoholic fermentation and that during this stage the predominant adsorptive action is observed, which determines the removal potential of the yeast. This mechanism was also confirmed for ochratoxin A (*22*).

During the alcoholic fermentation of grape juice, the technology of 'battonage' is frequently used at the end of the fermentation process (*23*). This involves mixing yeast lees in wine in order to obtain wines of higher quality. During this process, mannoproteins are released, and it is at this stage that the main adsorption of chemical contaminants is observed (*24*,*25*).

The adsorption activity of yeast lees is notably different among strains, and because of the cell wall structure it is clear that the physicochemical conditions, especially pH, affect the adsorption ratio (*18*,*24*,*25*). However, not only strain properties, but also differences in the binding affinity of pesticides, are important factors (*19*,*26*).

Removal of pesticides by degradation is a less common mechanism. Cabras and Angioni (*1*) showed that yeasts have the ability to degrade certain pesticides belonging to the pyrethroid class and certain insecticides belonging to the class of the thiophosphates. A mixed degradation/adsorption action has also been shown for some agrichemicals. Cabras *et al.* (*27*) showed that during fermentation yeasts partially degraded quinoxyfen and adsorbed it completely.

In addition to yeasts affecting the concentration of pesticides in the medium, pesticides can also affect microorganisms under certain conditions. For example fenhexamid did not affect alcoholic fermentation in studies performed by Cabras *et al.* (*28*), while a high concentration of pyrimethanil (10.0 mg/L) was found to significantly diminish the anaerobic growth of *Hanseniaspora uvarum* in YM medium (*29*). In some cases the presence of pesticides has been found to stimulate yeasts, particularly *Kloeckera apiculata*, which produced more alcohol (*30*). The presence of fungicides during alcoholic fermentation has also been demonstrated to affect secondary metabolite production, such as aroma compounds. Different pesticides have been shown to affect the aromatic profile of red and white wines (*2*,*31–34*).

The aim of this study is to elucidate the interactions of the fungicides pyrimethanil and fenhexamid with two *S. cerevisiae* wine yeast strains, one commercial and one isolated from a spontaneous fermentation, under stationary and fermentative conditions in synthetic media.

Materials and Methods

Yeast strains

The yeasts used in this study were a commercially available *Saccharomyces cerevisiae* Lalvin EC–1118 (Lallemand, Montreal, Canada), as active dry yeast, and the yeast strain *S. cerevisiae* ZIM 1927, previously isolated from a spontaneous fermentation of cv. Malvasia grape must in 2001 and obtained from the culture collection of industrial microorganisms, University of Ljubljana, Ljubljana, Slovenia. The first strain was rehydrated as described by the producer (Lallemand), and then cultured anaerobically in yeast extract-malt extract (YM) medium (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone (by mass) obtained from Biolife, Italy) supplemented with 18 % (by mass) glucose (Merck, Darmstadt, Germany) at 28 °C for 24 h, with rotary shaking at 190 rpm. The *Saccharomyces cerevisiae* ZIM 1927 was a three-day-old culture, maintained on YPD agar (YPD Broth, Oxoid, Basingstoke, UK) and cultured in YM medium as described for Lalvin EC-1118.

Cultivation and medium

The fermentations were carried out under anaerobic conditions. The cultivations were performed in liquid YM with 18.0 % (by mass) glucose at pH=6.37, previously sterilized by membrane filtration (0.20 μm, Sartorius, Göttingen, Germany). A 300-mL fermentor contained the control medium of 279 mL of YM and 1 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Three stock solutions of pyrimethanil and fenhexamid (dissolved in DMSO) were prepared separately to reach three concentrations of the pesticide in 280-mL medium: 10.0, 1.0 and 0.1 mg/mL, and sterilized by membrane filtration (0.20 μm, Sartorius). The final pH of the medium was 6.37. The fermentations were performed in triplicate for each strain and concentration of both pesticides (0.0 mg/L (control), 0.1, 1.0 and 10.0 mg/L).

Culture conditions and sampling

The inocula of the yeasts were prepared as described above and added directly to the fermentors to give a concentration of 10^6 CFU/mL of the medium (cell count by haemocytometer). Fermentations were carried out at 20 °C and fermentation kinetics was followed by CO_2 release (mass loss). Cultures were shaken/mixed daily. The final sample was taken 7 days after the end of the fermentation process (less than 2 g/L of residual sugars) (*21*,*22*). A final sample was also collected for measurement of volatile acidity.

Biomass recovery in stationary assays

In stationary assays, the biomass produced during fermentation under anaerobic conditions in liquid YM, with 18.0 % (by mass) glucose at pH=6.37, was recovered by centrifugation (10 min, 11 200×*g*) and washed three times (10 min, 11 200×*g*) with sterile phosphate buffer (pH=6.37), previously sterilized by filtration (0.20 µm, Sartorius). Finally, the cells were resuspended in phosphate buffer to give a final concentration of 0.44 g/mL.

Stationary assays of pyrimethanil and fenhexamid removal by the biomass

The assays were performed in duplicate in tubes containing 5 mL of assay solution, consisting of 4.882 mL of phosphate buffer (pH=6.37) and 0.018 mL of stock solution of DMSO/pyrimethanil or fenhexamid (Sigma-Aldrich) to reach three respective concentrations of pesticide in the medium (10.0, 1.0 and 0.1 mg/L). To each tube, 0.1 mL of yeast biomass suspension (viable or non-viable cells) in phosphate buffer were added to give a final concentration of 0.0088 g/mL. After 7 days of contact between the biomass and the medium (*21,22,35–37*), the solution was centrifuged and the non-adsorbed amounts of pesticides in the medium were measured. Non-viable cells were prepared by the addition of sodium azide (Sigma-Aldrich) to the biomass suspended in phosphate buffer (0.025 % final volume fraction in the media) in order to exclude metabolic action and preserve the structural integrity of the cells (*21,38*).

Determination of fungicide residues

The extraction procedure and determination of pyrimethanil and fenhexamid residues in liquid yeast extract-malt extract (YM) medium was done using a gas chromatography-mass spectrometry system (GC–MS) and liquid chromatography-tandem mass spectrometry system (LC/MS/MS), respectively according to the methods described previously (*15,39*).

For the determination of pyrimethanil, in the GC-MS analysis HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness) was used. Injector temperature was 250 °C, ion source temperature was 230 °C, auxiliary temperature was 280 °C and quadrupole temperature was 150 °C. GC oven temperature was programmed from 55 °C (held for 2 min) to 130 °C at a rate of 25 °C/min (held for 1 min), then to 180 °C at rate 5 °C/min (held for 30 min), then to 230 °C at a rate of 20 °C/min (held for 16 min), then to 250 °C at a rate of 20 °C/min (held for 13 min), and finally to 280 °C at a rate of 20 °C/min (held for 20 min). The helium constant flow was 1.2 mL/min. The liner used was Agilent 5181-3316 (Agilent Technologies, Palo Alto, CA, USA).

The content of fenhexamid residues in methanol extract was analyzed using liquid chromatography (PE200, PerkinElmer, Waltham, MA, USA) coupled with triple quadrupole mass detector (3200 QTrap, Applied Biosystems MDS Sciex, Concorde, Canada). Turbo spray temperature was kept at 650 °C. The compounds were separated on a Gemini C18 column, 250×4 mm (Phenomenex, Torrance, CA, USA). Gradient elution was used for pesticide separation. Mobile phase A consisted of 75 % of 5 mM $HCOONH_4$ and 25 % methanol (by volume) with 0.1 % formic acid added and mobile phase B consisted of 5 % of 5 mM $HCOONH_4$ and 95 % methanol (by volume) with 0.1 % formic acid added. The initial conditions (100 % mobile phase A) were maintained for 5 min, then linear gradient was applied and, in 30 min, 100 % of mobile phase B was reached and maintained for 15 min. Conditioning of the column to the initial mobile phase A was carried out for 10 min. Data was collected in multiple reaction monitoring (MRM) mode (dwell time 5 ms) and for each compound two MRM transitions were monitored.

Determination of volatile acidity and fermentation dryness

After the cultivation was completed, the volatile acidity in the medium was determined according to the accredited methods in the Central Laboratory of the Agricultural Institute of Slovenia, Ljubljana, Slovenia. Volatile acidity was determined by titration of the distillate obtained by steam distillation. A total of 20 mL of sample, freed from carbon dioxide, was placed in the distillation flask and 0.5 g of tartaric acid was added. Distillation was done using steam distillation apparatus (Oenoextracteur Chenard, France). At least 250 mL of the distillate were collected. The distillate was titrated with 0.1 M sodium hydroxide solution, using phenolphthalein as an indicator. The volatile acidity expressed in grams of acetic acid per litre was calculated according to the volume of sodium hydroxide used in the titration (*40*).

Fermentations were considered to have reached dryness when the concentration of reducing sugars was lower than 2 g/L in the fermentation media. Fermentation dryness was monitored at the end of fermentation with Clinitest®, Bayer, Leverkusen, Germany.

Statistical analysis

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

Results and Discussion

Interactions in stationary assays

Stationary phase assays were executed in phosphate buffer at the same pH as in the YM medium used in fermentation trials, *i.e.* pH=6.37. This medium was chosen to avoid any other external influence on the interaction between the yeast cells and the fungicides. We decided to use a contact time of 7 days (*21–25*). Viable and non-viable biomass were used, the latter being inactivated by sodium azide (*21,22,38*). The aims of the experiment were to determine the capacity of fresh biomass to remove the fungicides pyrimethanil and fenhexamid from synthetic media, the mechanism(s) of removal, (physicochemical or metabolic), and the effect of fungicide concentration on the interaction with two genetically different strains.

It can be seen in Table 1 that only 20 % of fenhexamid were removed by both strains when it was present at the initial concentration of 1.0 mg/L. Even at the initial concentration of 10.0 mg/L of fenhexamid, no difference

Table 1. Removal of pyrimethanil and fenhexamid in stationary assays by viable and non-viable biomass of *S. cerevisiae* strains ZIM 1927 and EC-1118

Initial concentration of fungicide in media/(mg/L)	Final concentration of fungicide in media/(mg/L)			
fenhexamid	ZIM 1927		EC-1118	
	viable cells	non-viable cells	viable cells	non-viable cells
0.1	0.08[a]	0.08[a]	0.08[a]	0.08[a]
1.0	0.83[a]	0.53[a]	0.57[a]	0.71[a]
10.0	5.76[a]	5.43[a]	7.08[a]	8.01[a]
pyrimethanil	ZIM 1927		EC-1118	
	viable cells	non-viable cells	viable cells	non-viable cells
0.1	0.05[a]	0.05[a]	0.05[a]	0.05[a]
1.0	0.43[a]	0.48[a]	0.41[a]	0.38[a]
10.0	5.10[a]	4.15[a]	3.12[a]	3.07[a]

All assays were performed in phosphate buffer, pH=6.37, at 20 °C for 7 days

Concentration of viable and non-viable yeast biomass added in tubes was 8.8 mg/mL

Non-viable cells: cells inactivated with 0.0025 % sodium azide

Mean values with the same superscript letter in the same line for the same strain do not differ significantly at $p \leq 0.05$

The data reported are average values of two independent replicates

between viable and non-viable cells of both strains was found. These results suggest that fenhexamid is removed from the media by adsorption and not by degradation since no significant difference between the removal potential of viable and non-viable cells could be observed. At the highest concentration of fenhexamid added (10.0 mg/L), viable and non-viable cells of EC-1118 removed 29.2 and 19.9 % of fenhexamid, respectively, whereas ZIM 1927 was able to remove 42.2 and 45.7 %, respectively. Other authors (*21,24,25,41*) have also observed a strain-dependent adsorption potential, as found in our assays.

The results obtained for pyrimethanil were similar to those for fenhexamid, as shown in Table 1. No significant differences in the removal potential between viable and non-viable cells suggest that the removal of pyrimethanil also involves adsorption. At the initial concentration of 0.1 mg/L, the removal was 50 % with both strains, whether viable or non-viable, and roughly similar results were obtained when the initial concentration was 1.0 mg/L. At the highest initial concentration studied (10.0 mg/L), viable and non-viable cells of ZIM 1927 removed 49.0 and 58.5 %, respectively, whereas EC-1118 had a higher potential for removal of up to 68.8 and 69.3 %, respectively.

Overall, it can be observed from these results that, independent of the strain and type of fungicide and its concentration, the removal of fungicide from synthetic media involves adsorption on yeast biomass and is not a consequence of metabolic degradation. This is similar to the previously obtained results (*42*). We observed that removal potential is strain dependent, but environmental conditions such as pH, temperature and the chemical composition of the media have also been shown to have a strong impact on binding capacities (*1,38*).

Fermentation kinetics in growth assays

The fermentation kinetics of the two strains varied in the YM media containing 18.0 % (by mass) of glucose at 20 °C. The duration of fermentations to dryness was 16 days for ZIM 1927 and 25 days for EC-1118 (Figs. 1 and 2). Fermentation duration did not affect the ability of each strain to achieve dryness.

The effect of both pesticides on the fermentation kinetics of strain EC-1118 can be seen in Figs. 1a and 2a. In the case of fenhexamid (Fig. 2a), its effect can be seen by the irregularity of fermentation curves when the pesticide was added; however, all spiked fermentations reached dryness. As the initial fungicide concentration increased, the fermentation performance decreased. Similarly, the addition of pyrimethanil (Fig. 1a) produced an irregularity of fermentation curves. When the initial concentration of spiked pyrimethanil was relatively low (0.1 or 1.0 mg/L), its effect on fermentation kinetics was not strong, but in the case of the addition of high amount (10.0 mg/L), the lag phase was longer and the logarithmic phase much slower in comparison with the control. However, in all cases dryness was reached.

In the case of ZIM 1927, for which the intensity of fermentation rate was higher, the effect of pesticides was found to be stronger. When fenhexamid was added at the two lower concentrations (0.1 and 1.0 mg/L) (Fig. 2b), no effects could be seen. On the other hand, at the concentration of 10.0 mg/L, longer lag phase and a strong reduction

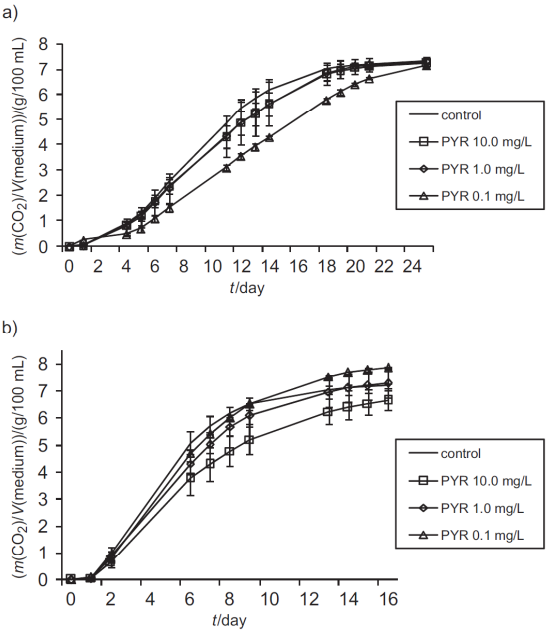

Fig. 1. Fermentation kinetics of *S. cerevisiae* strains: a) EC-1118 and b) ZIM 1927 in YM medium with 18.0 % glucose, pH=6.37, with the addition of pyrimethanil (PYR). The data reported are average values of three independent replicates

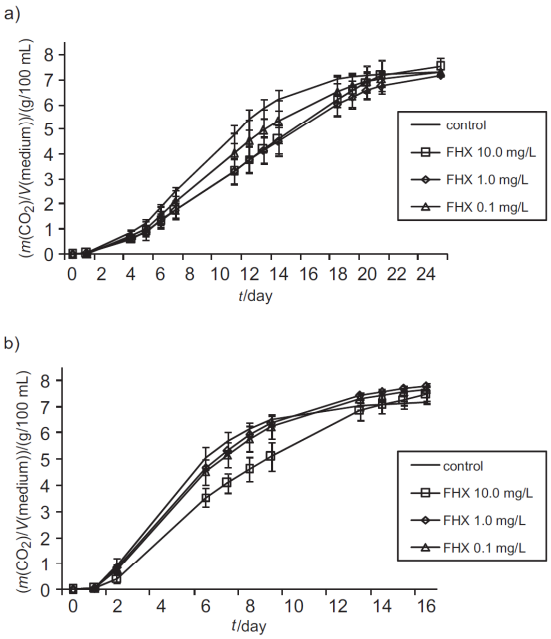

Fig. 2. Fermentation kinetics of *S. cerevisiae* strains: a) EC-1118 and b) ZIM 1927 in YM medium with 18.0 % glucose, pH=6.37, with the addition of fenhexamid (FHX). The data reported are average values of three independent replicates

in intensity of the logarithmic phase were observed. Despite this, in all cases, the fermentations reached dryness.

Similar trends can be seen in trials when pyrimethanil was added (Fig. 1b). Although no effect of the addition of pesticide at the two lower concentrations could be seen, a strong effect was present at the higher concentration of 10.0 mg/L. This is the only case when the fermentation did not reach dryness.

These results suggest that ZIM 1927 is more sensitive than EC-1118 to the effects of pesticides present in the media under the conditions tested. The intensity of fermentation rate could be the reason for higher sensitivity under stressful conditions. Additionally, irrespective of the strain, pyrimethanil had much stronger effect on the kinetics when compared to fenhexamid. These results are in agreement with previously published works on the effects of fenhexamid and pyrimethanil on yeast during fermentation (*28*,*29*). However, some pesticides such as tebuconazole were not found to have any effect on yeast kinetics during fermentation in synthetic media (*42*).

The effect of pyrimethanil and fenhexamid on the production of volatile acidity in growth assays

Volatile acidity production during alcoholic fermentation is an oenological parameter, and is a consequence of both genetic predisposition and environmental conditions. Stressful conditions in particular are known to induce the production of volatile acidity (*2*,*23*,*43*). The results in Table 2 show that, under our fermentation conditions, ZIM 1927 had significantly higher potential for volatile acidity production, which was 0.14 mg/L higher than for EC-1118 in the control fermentations. Fenhexamid was not found to have any effect on the volatile acidity production during alcoholic fermentation by either strain at any concentration studied. Likewise, pyrimethanil had no effect on volatile acidity production by either strain at the two lower concentrations (0.1 and 1.0 mg/L). However, significantly higher volatile acidity production was found with both strains at the highest concentration of pyrimethanil added, which means that pyrimethanil had a strong negative effect on both strains, as has also been shown in spontaneous fermentations (*29*). Although the production of volatile acidity significantly increased (by 21.6 %) in comparison with the control ZIM 1927, the strongest effect can be seen for EC-1118, where the increase in volatile acidity was 126.7 % higher than that of the control. These data show that the effect of pyrimethanil on volatile acidity production by EC-1118 was much greater than that of ZIM 1927.

Pyrimethanil and fenhexamid removal in growth assays

The results shown earlier in this paper suggest that the mechanism of removal of the fungicides pyrimethanil and fenhexamid by the yeast strains ZIM 1927 and EC-1118 does not involve metabolic degradation but rather is a consequence of physicochemical phenomena.

Extended contact with yeast lees following the completion of alcoholic fermentation was performed in order to determine the potential of the strains EC-1118 and ZIM 1927 to remove pyrimethanil and fenhexamid from the experimental media (*21*,*22*).

Table 2. Volatile acidity at the end of fermentation in YM with *S. cerevisiae* strains ZIM 1927 and EC-1118

Sample/strain	ZIM 1927	EC-1118	Sample/strain	ZIM 1927	EC-1118
control	$(0.74\pm0.01)^a$	$(0.60\pm0.02)^a$	control	$(0.74\pm0.01)^a$	$(0.60\pm0.02)^a$
PYR 0.1	$(0.69\pm0.01)^a$	$(0.63\pm0.03)^a$	FHX 0.1	$(0.70\pm0.05)^a$	$(0.54\pm0.07)^a$
PYR 1.0	$(0.69\pm0.00)^a$	$(0.66\pm0.07)^a$	FHX 1.0	$(0.67\pm0.02)^a$	$(0.57\pm0.04)^a$
PYR 0.0	$(0.90\pm0.08)^b$	$(1.36\pm0.05)^b$	FHX 10.0	$(0.70\pm0.07)^a$	$(0.65\pm0.09)^a$

control: fungicide not added
PYR: pyrimethanil added to the media at concentrations of 0.1, 1.0 and 10.0 mg/L
FHX: fenhexamid added to the media at concentrations of 0.1, 1.0 and 10.0 mg/L
Mean values with the same letter in the superscript in the same column do not differ significantly at $p\leq0.05$
The fermentation was carried out in YM containing 18.0 % glucose, pH=6.37, at 20 °C
The concentration of volatile acidity in media is expressed in mg/L of acetic acid
Data reported are average values of three independent replicates

In the case of pyrimethanil (Table 3), no difference between the two strains at any concentration could be seen, even at the highest initial concentration of 10.0 mg/L. Strain-related adsorption potential was not found in the growth assays, as was seen previously in the stationary assays. At all concentrations and for both strains there was roughly a 50 % removal. Similar results were observed for fenhexamid. At all initial concentrations studied, no significant differences between the two strains were found, and also in the case of fenhexamid, no strain-related adsorption potential was shown, as was found previously in the stationary tests.

Table 3. Removal of pyrimethanil and fenhexamid after the completion of alcoholic fermentation and prolonged contact for 7 days with yeast lees of *S. cerevisiae* strains ZIM 1927 and EC-1118

Initial concentration of fungicide/(mg/L)	Final concentration of fungicide in media/(mg/L)	
pyrimethanil	ZIM 1927	EC-1118
0.1	(0.06±0.01)[a]	(0.05±0.01)[a]
1.0	(0.50±0.01)[a]	(0.50±0.04)[a]
10.0	(5.21±0.07)[a]	5.08[a]*
fenhexamid	ZIM 1927	EC-1118
0.1	(0.06±0.00)[a]	(0.06±0.00)[a]
1.0	(0.52±0.02)[a]	(0.54±0.03)[a]
10.0	(4.86±0.09)[a]	(4.60±0.18)[a]

*average of two replicates
Mean values with the same letter in the superscript in the same column do not differ significantly at p≤0.05
YM contained 18.0 % glucose, pH=6.37, at 20 °C
Data reported are average values of three independent replicates

The more negative effects were observed when the concentrations of added fungicides were the highest. Under these conditions fermentation performance was reduced to a greater extent and a greater increase in volatile acidity production was observed. The toxicity of fenhexamid was shown to be lower than pyrimethanil. Since the concentration of yeast cells influences the removal of chemical contaminants from media (*21,24,25*), lower toxicity of fenhexamid might be the reason for its significantly higher removal.

Conclusions

The aim of this work was to elucidate the interactions of the fungicides pyrimethanil and fenhexamid with two genetically different *Saccharomyces cerevisiae* strains; one commercially available, Lalvin EC-1118, and ZIM 1927, a wild strain isolated from a spontaneous fermentation. Both strains were found to have the ability to remove both fungicides from synthetic media in stationary assays, and the results strongly suggest that the removal is a consequence of adsorption only, and not of degradation by metabolic action, since no significant difference between the assays with viable and non-viable cells was found. Strain-dependent adsorption potential was only found in stationary assays when conducted at the highest concentrations of the added pesticide, *i.e.* 10.0 mg/L.

No significant differences in the removal potential between the two strains at the same concentration of both fungicides could be observed during growth assays. However, pyrimethanil inhibited the completion of fermentation when added at a high concentration of 10.0 mg/L in growth assays with ZIM 1927 but not with EC-1118. The results suggest that pyrimethanil is more toxic to yeast cells during alcoholic fermentations; however, significant effects were only seen at the highest additions of pyrimethanil in relation to fermentation kinetics and volatile acidity production. Fenhexamid was less toxic to yeasts, and while it did not affect volatile acidity production at any concentration, when either yeast was used, its presence in the media impaired their fermentation kinetics.

The lower toxicity of fenhexamid, studied also at the highest added concentration in the media, seems to be the main reason for its higher removal. The removal capacity of the strains in synthetic media was found to be affected by both the toxicity of the chemical contaminant (in this case the fungicide) and different environmental conditions when determined in growth assays but not in stationary tests, so the results of these assays cannot be directly related (*21,22*). Further work is needed to demonstrate that the yeast-fungicide interactions observed in synthetic media can be found in grape must.

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2.1.3 A breeding strategy to harness flavor diversity of *Saccharomyces* spp. interspecific hybrids and minimize hydrogen sulfide production

Plemenilna strategija za dosego aromatske kompleksnosti in zmanjšanje produkcije H_2S pri medvrstnih hibridih *Saccharomyces* spp.

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Industrial food-grade yeast strains are selected for traits that enhance their application in quality production processes. Wine yeasts are required to survive in the harsh environment of fermenting grape must, while at the same time contributing to wine quality by producing desirable aromas and flavors. For this reason, there are hundreds of wine yeasts available, exhibiting characteristics that make them suitable for different fermentation conditions and winemaking practices. As wine styles evolve and technical winemaking requirements change, however, it becomes necessary to improve existing strains. This becomes a laborious and costly process when the targets for improvement involve flavor compound production. Here, we demonstrate a new approach harnessing preexisting industrial yeast strains that carry desirable flavor phenotypes – low hydrogen sulfide (H_2S) production and high ester production. A low-H_2S *Saccharomyces cerevisiae* strain previously generated by chemical mutagenesis was hybridized independently with two ester-producing natural interspecies hybrids of *S. cerevisiae* and *Saccharomyces kudriavzevii*. Deficiencies in sporulation frequency and spore viability were overcome through use of complementary selectable traits, allowing successful isolation of several novel hybrids exhibiting both desired traits in a single round of selection.

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RESEARCH ARTICLE

A breeding strategy to harness flavor diversity of *Saccharomyces* interspecific hybrids and minimize hydrogen sulfide production

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Abstract

Industrial food-grade yeast strains are selected for traits that enhance their application in quality production processes. Wine yeasts are required to survive in the harsh environment of fermenting grape must, while at the same time contributing to wine quality by producing desirable aromas and flavors. For this reason, there are hundreds of wine yeasts available, exhibiting characteristics that make them suitable for different fermentation conditions and winemaking practices. As wine styles evolve and technical winemaking requirements change, however, it becomes necessary to improve existing strains. This becomes a laborious and costly process when the targets for improvement involve flavor compound production. Here, we demonstrate a new approach harnessing preexisting industrial yeast strains that carry desirable flavor phenotypes – low hydrogen sulfide (H_2S) production and high ester production. A low-H_2S *Saccharomyces cerevisiae* strain previously generated by chemical mutagenesis was hybridized independently with two ester-producing natural interspecies hybrids of *S. cerevisiae* and *Saccharomyces kudriavzevii*. Deficiencies in sporulation frequency and spore viability were overcome through use of complementary selectable traits, allowing successful isolation of several novel hybrids exhibiting both desired traits in a single round of selection.

Introduction

Wine yeast improvement is a continuous process of fulfilling winemaking needs (Pretorius, 2000; Giudici *et al.*, 2005). Strain improvement strategies are numerous – often complementary to each other – and the choice among them is based on three factors: (1) the genetic nature of traits (monogenic or polygenic); (2) the knowledge of the genes involved (rational or blind approaches); and (3) the phenotypic trait requirement (Giudici *et al.*, 2005). Because of the lack of acceptance for the use of recombinant yeasts, only classical techniques such as clonal selection of variants, mutation and selection, and mating/hybridization are currently used to produce food-grade starter cultures (Pretorius, 2000; Schilter & Constable, 2002). Improvement of wine yeast properties means not only enhancing fermentation performance, but also optimizing the production of yeast secondary metabolites such as volatile aroma compounds. Some compounds are detrimental to wine quality, and therefore, their production should be minimized. A well-studied example is hydrogen sulfide (H_2S), a yeast metabolite that imparts rotten-egg off-flavor in wine (Zambonelli *et al.*, 1984; Tezuka *et al.*, 1992; Omura *et al.*, 1995; Hansen & Kielland-Brandt, 1996; Mendes-Ferreira *et al.*, 2002; Nowak *et al.*, 2004). On the other hand, enhancing the formation of positive aroma compounds belonging to chemical families such as aldehydes, ketones, alcohols, acids, and esters by wine yeasts has also been a priority (Pretorius, 2000; Pretorius & Bauer, 2002; Fleet, 2003).

A recent approach to the improvement of wine yeast strains involved generation of interspecies hybrids within the *Saccharomyces sensu stricto* group (Bellon *et al.*, 2011). Natural interspecies hybrids were previously isolated from

wine fermentation (Gonzalez *et al.*, 2006), while the lager yeast *S. pastorianus* is in fact a natural hybrid of *Saccharomyces cerevisiae and S. bayanus* (Sipiczki, 2008; Libkind *et al.*, 2011). Generally considered sterile and a reproductive 'dead-end,' *Saccharomyces* interspecies hybrids can potentially undergo further hybridization (de Barros Lopes *et al.*, 2002). Indeed, through application of molecular identification methods, natural double (Sipiczki, 2008) and triple hybrids (*S. cerevisiae* × *Saccharomyces uvarum* × *Saccharomyces kudriavzevii*) (Masneuf *et al.*, 1998; de Barros Lopes *et al.*, 2002; Gonzalez *et al.*, 2006) were identified.

Our aim, therefore, was to demonstrate that existing *Saccharomyces* interspecies hybrids can be used in breeding programs with characterized *S. cerevisiae* mutants, to develop novel industrial wine yeast strains with improved aroma traits.

Materials and methods

Yeast strains

All the yeast strains were obtained from The Australian Wine Research Institute (AWRI) culture collection. Yeast cultures were maintained on solid yeast peptone dextrose (YPD) agar plates.

Media and grape musts

YPD was composed of 2% D-glucose, 1% yeast extract, and 2% peptone and solidified with 2% agar. Sporulation media (SM) were composed of 1% potassium acetate, 0.05% D-glucose, 0.1% yeast extract, and 2% agar. BiGGY agar (Oxoid, Australia) was prepared using manufacturer's instructions. YP-Galactose was prepared with 2% D-galactose, 1% yeast extract, and 1% peptone and solidified with 2% agar at different pH values. Two filter-sterilized Chardonnay grape juices were used for wine fermentation (CHS and CH07). Both juices were filter-sterilized using a 0.65-μm/0.22-μm cartridge (Sartorius, Germany).

Screening for parental strains

The screening of candidate parental strains was performed in two stages. First, the strains were grown on BiGGY agar, which provides a qualitative indication of H_2S production potential (Giudici & Kunkee, 1994). The chemically mutagenized low-H_2S strains included in this study were isolated by Cordente *et al.* (2009) using this medium.

The second stage was a laboratory fermentation trial with sterile-filtered Chardonnay juice (CHS). Yeast precultured sequentially in YPD and CHS were inoculated at 10^6 cells mL^{-1} in 200 mL CHS, with alcoholic fermentation conducted in 250-mL flasks at 17 °C. Fermentation progress was followed by CO_2 weight loss, measured every 24 h. Ferments were considered complete when CO_2 release was lower than 1 g L day^{-1}, and the concentration of reducible sugars was lower than 2 g L^{-1} (Clinitest, Bayer, Germany).

Sporulation, tetrad dissection, and viability of spores

Strains were sporulated on solid SM media for 5 days at 28 °C. Spores were obtained using a standard zymolyase protocol (Burke *et al.*, 2000). Using a micromanipulator microscope (Singer, UK), the spores were separated and distributed on an YPD plate. Viability of spores was evaluated after incubation of spores for 3 days, at 28 °C.

Screening for complementary phenotypic markers of parental strains

To develop discriminating methodologies for selection of hybrids, we performed a range of tests to determine complementary phenotypic markers. We tested the parental strains for ability to use different carbon sources in liquid media (glucose, fructose, saccharose, mannose, and galactose) at 6% w/v, except for raffinose at 12% w/v, with 1% yeast extract and 1% peptone (Kreger-van Rij, 1984). We also tested their ability to grow under high concentrations (0, 150, 200, and 250 mg L^{-1}) of SO_2 and ethanol (8, 10, 11, 12, and 13% v/v) in YPD broth at pH 3.5, after 72 and 48 h, respectively. Finally, the ability for growth under low pH (from 2.5 to 2.9) conditions was estimated in YPD broth after 24 h. All the liquid media were sterilized prior to use, by membrane filtration (0.22 μm, Sartorius, Germany), and media were inoculated at a concentration of 10^6 cells mL^{-1}. The strains were also tested for growth at 37 °C on YPD solid agar media after 24 h. Strains were spotted on solid media after overnight growth in YPD broth at a concentration of 3×10^3 cells per spot. All the tests were performed in triplicate.

Mass-mating and selection of potential hybrids

Yeast strains AWRI 1116 (interspecies hybrid 1, strain A), 1539 (interspecies hybrid 2, strain B), and 1640 (low-H_2S strain, strain C) were inoculated on solid SM for 5 days at 28 °C, and cells were incubated with zymolyase for 1 h at 30 °C on a rotor incubator. Then, 0.1 mL of sterile 0.5-mm glass beads was added and incubated on a rotor at 30 °C for 1 h. Asci were then disrupted by addition of 1 mL sterile water and vortex for 1–2 min (Burke *et al.*,

2000). Spores were collected, washed in sterile H_2O, mixed, and inoculated in YPD broth. After 7 days at 20 °C with rotary shaking (120 r.p.m.), the media were spread onto selective plates (200 µL per plate).

For hybrids of AWRI 1116 × 1640 (A × C), YP-Galactose solid media at pH 3.1 were used, and hybrids were isolated after 4 days at 28 °C. Hybrids of AWRI 1539 × 1640 (B × C) were selected on YP-Galactose solid media after overnight growth at 37 °C. All the putative hybrids were analyzed (in duplicate) for H_2S production on BiGGY plates (Giudici & Kunkee, 1994). Hybrids of AWRI 1539 × 1640 and of AWRI 1116 × 1640 showing lower H_2S production than AWRI 1539 and AWRI 1116, respectively, were submitted for further genotypic identification.

Molecular analysis of hybrids

Genomic DNA extraction of yeast strains was performed using glass beads for cell wall breakage (Burke *et al.*, 2000). ITS-PCR-RFLP (McCullogh *et al.*, 1998) was performed to detect inheritance of *S. kudriavzevii* genomic regions containing ribosomal DNA regions, while transposon-PCR (Ness *et al.*, 1993) was to differentiate the *S. cerevisiae* component of parental strains, and their progeny. A 1.5-kb fragment of the *S. kudriavzevii MET10* gene was amplified by PCR with specific primers, and a 1.4-kb fragment of the *S. cerevisiae MET10* gene was amplified by PCR with specific primers. All *MET10* PCR products were cleaned using the QIAquick PCR Purification kit (QIAGEN, Australia) and used as templates for the sequencing reaction. Sequencing was carried out on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Australia) by the Australian Genome Research Facility Ltd sequencing service (Adelaide). All analyses of genotypic fingerprints were performed on the screening cartridge of the QIAxcel capillary electrophoresis system (QIAGEN).

Alcoholic fermentations of Chardonnay grape juice with H_2S detection

Laboratory-scale fermentations in filter-sterilized Chardonnay juice CH07 were carried out in the same conditions used for selection of parental strains. H_2S liberated during fermentation was quantitated by a trap-based method, using precision gas detector tubes (Kitagawa, Japan).

Analysis of principal nonvolatile compounds

The wines produced from Chardonnay CH07 were analyzed for glucose, fructose, ethanol, glycerol, and acetic, citric, malic, succinic, lactic, and tartaric acid by HPLC using a Bio-Rad HPX-87H column as described previously (Nissen *et al.*, 1997). Sulfite (SO_2) measurements were taken using the Flow Injection analysis using the Lachat Instrument Quick Chem(r) FIA + 8000 series (Cordente *et al.*, 2009).

Gas chromatography–mass spectrometry (GC–MS) analysis

All analyses were performed on an Agilent 7890 gas chromatograph equipped with Gerstel MPS2 multipurpose sampler and coupled to an Agilent 5975C VL mass selective detector. Instrument control and data analysis were performed with Agilent G1701A Revision E.02.00 ChemStation software.

Nonchromatographic volatile fingerprinting

Nonchromatographic volatile fingerprinting of wine samples was performed by Metabolomics Australia (Adelaide) as follows. Each sample was prepared in duplicate by diluting 1 : 4 with 10% potassium hydrogen tartrate, pH adjusted 3.5. The gas chromatograph was fitted with a deactivated guard column (Restek 5 m × 180 µm × 0.18 µm). Helium (Ultra High Purity) was used as the carrier gas at a flow rate of 1.3 mL min^{-1} in constant flow mode. The oven temperature held constant at 200 °C for the duration of the analysis.

The sample was heated to 40 °C for 5 min with agitation. A volume of 2.5 mL of the headspace was removed using a heated (55 °C) syringe. The content of the syringe was then injected into a Gerstel PVT (CIS 4) inlet fitted with a Tenax TA inlet liner cooled to 15 °C using solvent vent mode (pressure 2 psi for 2 min). Following capture of analytes on the Tenax liner, the injector is heated to 330 °C at 12 °C s^{-1} (pressure 12.104 psi).

The mass spectrometer quadruple temperature is set at 150 °C, the source was set at 250 °C, and the transfer line is held at 280 °C. Positive ion electron impact spectra at 70 eV are recorded in scan mode with a mass range of 50–400 and 4 scans s^{-1}.

Raw data were exported from ChemStation (Agilent G1701A Revision E.02.00 ChemStation Software). Mass spectra were summed (scan 173–scan 391) and binned (0.5 mass units) using R (version 2.9.2).

Targeted analyses of volatile compounds

Targeted analyses of fermentation-derived higher alcohols, acetate-, and ethyl esters were performed by Metabolomics Australia (Adelaide) on wine samples by GC–MS using a stable isotope dilution assay as previously

described (Siebert *et al.*, 2005), with modifications to sample concentration and introduction. Briefly, the SPME (solid-phase microextraction) sample concentration and introduction technique was replaced with a large volume headspace using a cooled Tenax liner, as described for the nonchromatographic volatile fingerprinting method mentioned earlier.

Further modifications to the method of Siebert *et al.* (2005) included the following: Wine samples were prepared in two dilutions 1/20 and 3/10 with model wine (13.8% ethanol, 10% potassium hydrogen tartrate, pH 3.5). The gas chromatograph was fitted with a 30 m × 0.18 mm Resteck Stabilwax-DA that has a 5 m × 0.18 mm retention gap. Helium was used as the carrier gas with linear velocity of 24.6 cm s^{-1} and flow rate of 0.78 mL min^{-1} in constant flow mode. The oven temperature started at 33 °C was held at this temperature for 4 min, then increased to 60 °C at 4 °C min^{-1}, then heated at 8 °C min^{-1} to 230 °C, and held at this temperature for 5 min.

Prior to injection, the inlet was cooled to 0 °C with liquid nitrogen. While maintaining 0 °C, the sample was introduced to the inlet at 25.0 μL s^{-1} (penetration, 22.0 mm) using split mode (split ratio, 33 : 1; split flow, 25.78 mL min^{-1}). Following capture of analytes on the Tenax liner, the injector was heated to 330 °C at 12 °C min^{-1} (pressure, 24.6).

The mass spectrometer quadrupole temperature was set at 150 °C, the source was set at 250 °C, and the transfer line was held at 280 °C. All data processing was performed on Agilent G1701A Revision E.02.00 ChemStation software.

Statistical analysis

The analyzed data from alcoholic fermentation trials were studied by one-way ANOVA (Microsoft Office Excel 2003). The statistical level of significance was set at $P \leq 0.05$. The means were compared with the Tukey's honestly significant different test. Volatile fingerprinting ASCII data matrices were imported into The Unscrambler (Camo, Version 9.8), and principal component analysis (PCA) was performed.

Results

Selection of parental strains and screening for complementary phenotypic markers

The first industrial, food-grade, low-H_2S-producing wine yeast strains were obtained by chemical mutagenesis of the widely used commercial strain Maurivin PDM (Cordente *et al.*, 2009). To develop strains with improved traits for wine production, we hybridized the strain displaying the lowest H_2S production (AWRI 1640, strain C) from that study, with yeast strains known to produce relatively high concentrations of positive flavor compounds.

These strains, AWRI 1116 and 1539 (strains A and B, respectively), both natural interspecific hybrids of *S. cerevisiae* and *S. kudriavzevii*, were used to ferment Chardonnay juice CHS. Chemical analysis of the wines showed that the overall production of volatile fermentation products was 37% and 22% higher for AWRI 1116 and 1539, respectively, in comparison with the low-H_2S strain AWRI 1640 (Table 1). Both AWRI 1116 and 1539 produced increased levels of acetate esters (excluding ethyl acetate) and higher alcohols than AWRI 1640. Of note, both AWRI 1116 and 1539 produced significant higher concentrations of 2-methylpropyl acetate (banana, fruity aroma) and 2- and 3-methylbutyl acetate (banana aroma), while producing less ethyl acetate (nail polish/solvent aroma). In the case of AWRI 1539, this strain also produced significant higher levels of 2-phenylethyl acetate (rose aroma) than AWRI 1640.

Qualitative assessment of H_2S production on BiGGY agar confirmed the higher potential for H_2S production by AWRI 1116 and 1539. Both strains displayed a dark

Table 1. Volatile compounds (μg L^{-1}) produced by parental strains AWRI 1116, 1539, and 1640 at the end of fermentation in a Chardonnay juice (CHS)

	Strain		
Volatile compound	1116 (A)	1539 (B)	1640 (C)
Ethyl propanoate	280^{ab}	129^{b}	1074^{a}
Ethyl 2-methylpropanoate	92^{ab}	100^{a}	50^{b}
Ethyl butanoate	88^{a}	150^{b}	189^{c}
Ethyl 2-methylbutanoate	23^{a}	21^{a}	39^{a}
Ethyl 3-methylbutanoate	21^{a}	20^{a}	19^{a}
Ethyl hexanoate	193^{b}	261^{a}	235^{ab}
Total ethyl esters	697	681	1606
Ethyl acetate	73008^{a}	42143^{b}	99556^{c}
2-methylpropyl acetate	53^{a}	55^{a}	36^{b}
2-methylbutyl acetate	99^{a}	74^{b}	49^{c}
3-methylbutyl acetate	783^{a}	651^{a}	312^{b}
2-phenylethyl acetate	189^{ab}	316^{a}	98^{b}
Hexyl acetate	27^{ab}	32^{a}	19^{b}
Total acetate esters*	1151	1128	514
2-methylpropanol	75219^{a}	72771^{a}	34282^{b}
Butanol	243^{a}	179^{a}	1115^{a}
2-methylbutanol	86938^{a}	57774^{b}	36123^{c}
3-methylbutanol	220304^{a}	191513^{a}	112810^{b}
Hexanol	1569^{a}	1545^{a}	1680^{a}
Total alcohols	384273	323752	186010

Results are the mean of two independent replicates. Standard deviations were typically about 10% and never exceeded 20%. Means with the same letter are not significantly different from each other (Tukey's test, $P < 0.05$).
*Excluding ethyl acetate.

brown phenotype in this medium, while strain AWRI 1640 had a white phenotype (data not shown).

Spore viability was evaluated for each of the three parental strains, and we were unable to isolate a viable spore among 18 tetrads; thus, we were reliant upon mass-mating.

To discriminate hybrids from the parental strains, complementary phenotypic markers were sought (Table 2). Carbon source utilization screening revealed that the parental low-H_2S-producing strain AWRI 1640 was not able to grow on galactose. The interspecies hybrid AWRI 1539 could not grow at 37 °C, while AWRI 1116 grew slowly at low pH in comparison with AWRI 1640. Thereafter, we designed media and growth conditions for selective isolation of hybrids by mass-mating (see Materials and methods).

A total of 31 potential hybrids of AWRI 1539 × 1640 (B × C) and 65 potential hybrids of AWRI 1116 × 1640 (A × C) were isolated from selective plates. The hybrids were screened for H_2S production on BiGGY agar plates. A total of 20 B × C and 24 A × C potential hybrid colonies were lighter in color compared to AWRI 1539 and AWRI 1116, respectively (data not shown), and were chosen for further molecular characterization.

Table 2. Identification of complementary phenotypes for parental strains AWRI 1116, 1539, and 1640

	Strain		
Treatment	1116 (A)	1539 (B)	1640 (C)
Temperature			
37 °C	+	–	+
Carbon source			
Glucose	+	+	+
Fructose	+	+	+
Mannose	+	+	+
Saccharose	+	+	+
Raffinose	+	+	+
Galactose	+	+	–
pH			
2.5	–	–	–
2.6	–	–	+
2.7	–	+	+
2.8	+	+	+
2.9	+	+	+
Ethanol (v/v%)			
8	+	+	+
10	+	+	+
11	+	+	+
12	–	–	+
13	–	–	–
SO_2 (mg L^{-1})			
0	+	+	+
150	–	+	+
200	–	+	–
250	–	–	–

Growth (+) and absence of growth (–) are shown.

Confirmation of hybrid genotypes

Two techniques were applied to identify and discriminate between parental strains and hybrids. ITS-PCR-RFLP analysis (Fig. 1a) confirmed the presence of *S. cerevisiae* and *S. kudriavzevii* ribosomal DNA regions within the genomes of three putative B × C hybrids (AWRI 1808, 1809, and 1810). By transposon-PCR (Fig. 1b), the three previously identified B × C hybrids and 2 A × C hybrids (AWRI 1811 and 1812) displayed differential fingerprints that were intermediate to their relevant parental strains.

Further molecular characterization was applied to the five selected hybrids by sequencing the *S. cerevisiae MET10* gene, which encodes for the α-subunit of the sulfite reductase enzyme. AWRI 1640 has a heterozygous point mutation (G176A) in *MET10*, which is responsible for the low-H_2S phenotype of the strain (Cordente *et al.*, 2009). All five hybrids were found to contain this mutation, along with extra copies of wild-type *S. cerevisiae MET10* alleles. All B × C hybrids contained three extra *MET10* alleles, one of them coming from the AWRI 1640 parental strain and the other two from the *S. cerevisiae* genome of AWRI 1539. In the A × C hybrids, two extra *MET10* alleles were found, one from AWRI 1640 and the other from AWRI 1116. In addition, the *S. kudriavzevii MET10* gene was found to be present in all five novel low-H_2S-producing hybrids (Fig. 2).

Laboratory-scale fermentations of novel hybrids in Chardonnay grape juice

The five hybrid strains were further characterized in a 200 mL fermentation experiment in Chardonnay juice CH07. The A × C hybrids AWRI 1811 and 1812 fermented at a similar rate to AWRI 1640, which was slightly faster than AWRI 1116 (Fig. 3). On the other hand, the B × C hybrids AWRI 1808, 1809, and 1810 (Fig. 4) showed intermediate fermentation rates between their parental strains AWRI 1640 and 1539, the latter being the fastest.

The major nonvolatile compounds were analyzed at the end of fermentation, and some differences were observed between strains (Table 3). All the hybrids produced significant lower concentrations of acetic acid (< 0.1 g L^{-1}) than their parental strains. AWRI 1640 produced significantly higher concentrations of glycerol than AWRI 1116 and 1539 parental strains, and this was also the case for all of the novel hybrids.

Cumulative production of H_2S was measured throughout fermentation, and SO_2 production was analyzed at

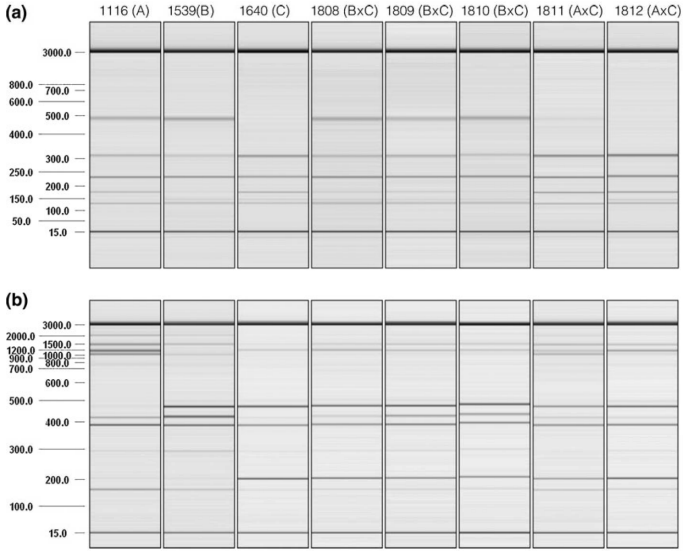

Fig. 1. ITS-PCR-RFLP (a) and transposon-PCR (b) analysis of parental strains and novel hybrids using the QIAxcel capillary electrophoresis system (QIAGEN). From left to right: AWRI 1116 (A), 1539 (B), 1640 (C), B × C hybrids 1808, 1809, 1810, and A × C hybrids 1811, 1812.

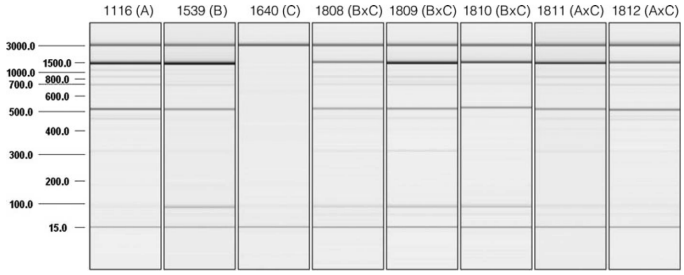

Fig. 2. Amplification of *Saccharomyces kudriavzevii MET10* (1500 bp amplicon), and analysis using the QIAxcel capillary electrophoresis system (QIAGEN). From left to right: AWRI 1116 (A), 1539 (B), 1640 (C), B × C hybrids 1808, 1809, 1810, and A × C hybrids 1811, 1812.

the end of fermentation. Both AWRI 1539 and 1116 were found to be relatively high H_2S producers (975 and 850 mg L^{-1}, respectively), while producing minimal concentrations of SO_2 (Table 3). On the other hand, no H_2S production was observed for AWRI 1640; however, high levels of total SO_2 (146 mg L^{-1}) were accumulated, in accordance with Cordente *et al.* (2009). A × C hybrids AWRI 1811 and 1812 behaved similarly to AWRI 1640, with low or even nondetectable levels of H_2S, respectively, and a high SO_2 production phenotype. On the other hand, B × C hybrids AWRI 1808, 1809, and 1810 showed an intermediate H_2S and SO_2 production phenotype when compared to their parental strains.

Volatile fingerprints of wines made with novel hybrids

Utilizing a nonchromatographic analytical method, volatile fingerprints were obtained for each of the three parental strains and the five novel hybrids. PCA of the binned mass spectroscopy data reflects the fermentation products profiles (Table 1) in that wines made with AWRI 1116 and AWRI 1539 cluster more closely together, while AWRI 1640 is clearly separated from them (Fig. 5). Wines made with each of the novel hybrids clustered intermediate to their respective parental strains. Interestingly, the A × C hybrids exhibit a volatile fingerprint

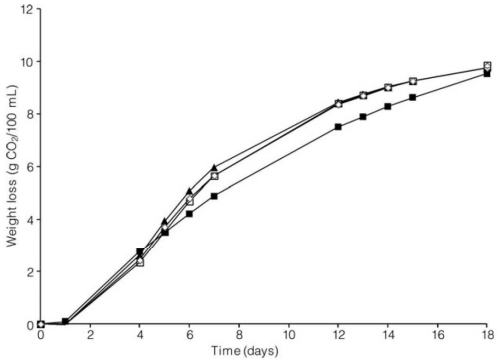

Fig. 3. Fermentation kinetics (depicted as cumulative weight loss). Parental strains AWRI 1116 (■) and 1640 (□), and A × C hybrids AWRI 1811 (▲) and AWRI 1812 (◇) in sterile Chardonnay (CH07) grape juice. Standard deviations did not exceed 5%.

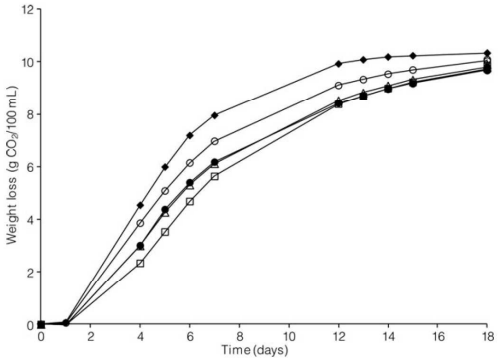

Fig. 4. Fermentation kinetics (depicted as cumulative weight loss). Parental strains AWRI 1539 (♦) and 1640 (□), and B × C hybrids AWRI 1808 (Δ), AWRI 1809 (●), and AWRI 1810 (○) in sterile Chardonnay (CH07) grape juice. Standard deviations did not exceed 5%.

that clusters closer with that of the AWRI 1640 parental strain, while the B × C hybrids clustered more closely to the AWRI 1539 parental strain.

Discussion

Although natural and laboratory-bred interspecies hybrids exhibit interesting oenological traits such as intense and complex flavor production (Swiegers *et al.*, 2009; Bellon *et al.*, 2011), they have a low breeding potential and are generally considered as an end point for further breeding (Hawthorne & Philippsen, 1994; Marinoni *et al.*, 1999; Greig *et al.*, 2002; Sebastiani *et al.*, 2002).

In this study, we demonstrate that it is possible to breed interspecies hybrids to develop novel wine yeast strains with desired oenological traits. This was possible through the combination of a mass-mating strategy, the use of complementary phenotypic markers for the selection of hybrids, and the simplicity of the method used for screening of H_2S production. It is important to note that through use of a phenotype such as thermotolerance for selection, shown to be polygenic in industrial strains of *S. cerevisiae* (Marullo *et al.*, 2009), we may have excluded some hybrids with desirable properties.

Characterization of the five selected hybrid strains using molecular biology techniques suggested a mixed inheritance of parental genomes. All hybrids contain at least a part of the genome of each of the parental strains: *S. kudriavzevii* and *S. cerevisiae* from the flavor-active parents (either AWRI 1539 or AWRI 1116), and *S. cerevisiae* from the low-H_2S-producing strain (AWRI 1640). All five selected hybrids contained both *MET10* alleles from the low-H_2S-producing strain and at least one *S. cerevisiae* allele from the flavor-active parent, in addition to the *S. kudriavzevii MET10* allele. Genome stabilization in *Saccharomyces* interspecies hybrids has been shown to involve extensive translocation, segmental duplication, and even chromosomal loss (Antunovics *et al.*, 2005); thus, it is reasonable to expect our novel hybrids to display extensive aneuploidy.

The presence of *MET10* G176A allele in all five hybrids strongly indicated that this mutation was responsible for the low-H_2S phenotype of the strains, as demonstrated for the AWRI 1640 parental strain (Cordente *et al.*, 2009). From these results, it is also clear that the G176A allele has a strong dominant effect, not only over other *S. cerevisiae* alleles, but also over those from *S. kudriavzevii*. Therefore, AWRI 1640 could be used in the future as a tool for production of new hybrids, with low H_2S production combined with other important industrial traits.

The main concern of the low-H_2S-producing strains is their high SO_2 production during fermentation (Cordente *et al.*, 2009), which can cause the inhibition of malolactic fermentation by lactic acid bacteria in wines (Rankine & Bridson, 1971). With our strategy, the risk of producing high amounts of SO_2 could be reduced. Three of the five selected strains showed a significant reduction in SO_2 levels when compared with the low-H_2S parental strain. In this regard, the most oenologically suitable strain was found to be the B × C hybrid AWRI 1810, which produced the lowest amount of SO_2 (17 mg L^{-1}) of all novel hybrids and only produced 15% as much H_2S as AWRI 1539. In all wines, SO_2 was only present in the bound form, and no free SO_2 could be detected, the latter being the most antimicrobially active. This suggests that both B × C hybrids AWRI 1808 and 1809 could also be utilized

Table 3. Main fermentation parameters at the end of fermentations in 200 mL of sterile Chardonnay grape juice (CH07)

Strain	Residual sugar (g L^{-1})	Glycerol (g L^{-1})	Citric acid (g L^{-1})	Malic acid (g L^{-1})	Succinic acid (g L^{-1})	Tartaric acid (g L^{-1})	Lactic acid (g L^{-1})	Acetic acid (g L^{-1})	Ethanol (%) (v/v)	H_2S (mg L^{-1})	Total SO_2 (mg L^{-1})
1116 (A)	0.0^{b}	6.23^{b}	0.14	1.48^{e}	2.29^{ac}	3.29^{b}	0.12^{bc}	0.41^{e}	14.3^{a}	850^{a}	Vnd
1539 (B)	0.0^{b}	6.19^{b}	0.15	1.31^{b}	2.32^{a}	1.86^{a}	0.10^{b}	0.22^{b}	13.5^{b}	975^{a}	5^{e}
1640 (C)	2.6^{a}	7.64^{a}	0.15	1.95^{a}	2.25^{a}	2.18^{ac}	0.08^{a}	0.17^{a}	14.3^{a}	nd	46^{a}
1808 (B × C)	2.4^{a}	7.42^{a}	0.16	2.54^{c}	2.19^{a}	1.98^{a}	0.14^{c}	0.03^{c}	14.3^{a}	18^{c}	80^{b}
1809 (B × C)	3.2^{a}	7.32^{a}	0.14	2.26^{d}	2.17^{a}	2.35^{ac}	0.13^{bc}	0.09^{d}	14.2^{a}	12^{d}	67^{c}
1810 (B × C)	1.9^{a}	6.76^{c}	0.15	1.75^{a}	1.94^{b}	2.20^{ac}	0.11^{b}	0.08^{d}	13.7^{b}	142^{b}	7^{d}
1811 (A × C)	2.2^{a}	7.84^{a}	0.15	2.10^{f}	2.41^{c}	2.26^{ac}	0.10^{bc}	0.08^{d}	14.2^{a}	5^{e}	45^{a}
1812 (A × C)	3.0^{a}	6.77^{c}	0.16	2.75^{c}	2.00^{b}	1.97^{a}	0.20^{d}	0.02^{c}	14.1^{a}	nd	52^{a}

Average values of three independent repeats. All the standard deviations for triplicates are less than 10%. Means with the same letter are not significantly different from each other (Tukey's test, $P < 0.05$).
n.d., nondetectable.

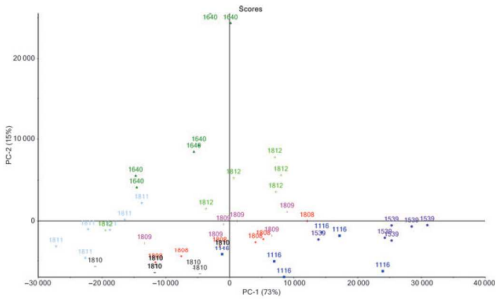

Fig. 5. Volatile fingerprinting of wines made with novel hybrids. PCA of the binned mass spectroscopy data for wines produced by yeast strains AWRI 1116 (A), 1539 (B), 1640 (C), B × C hybrids 1808, 1809, 1810, and A × C hybrids 1811, 1812. PC1 explains 73% of variation in the dataset, and PC2 15%.

in commercial wine production in spite of their relatively high SO_2 production (80 and 67 mg L^{-1}, respectively), if malolactic fermentation is not practiced (Ribereau-Gayon *et al.*, 2006).

On the basis that fermentation kinetics and SO_2 and H_2S production by the two A × C hybrids were more similar to AWRI 1640 than to the AWRI 1116 strain, we can speculate that there has been a major contribution of the genetic background from AWRI 1640. This was consistent with the fermentation product 'fingerprints' as PCA shows that AWRI 1811 and 1812 wines clustered closer to the low-H_2S *S. cerevisiae* parental strain.

As for the B × C hybrids, AWRI 1808 and 1809 showed intermediate fermentation kinetics and SO_2/H_2S production between both parental strains; on the other hand, AWRI 1810 had faster kinetics and produced a similar amount of SO_2 as the AWRI 1539 parental strain, perhaps reflecting a major contribution of the genetic material from this strain. The PCA of volatile fingerprints was consistent with the other observations for AWRI 1808 and 1809, as they clustered intermediate to the two parental strains. The volatile fingerprint for AWRI 1810, on the other hand, was least similar to either parental strain.

The genome sequence for the commercial wine yeast strain VIN7 (of which AWRI 1539 is an isolate) was recently shown to be comprised of diploid *S. cerevisiae* chromosomes and an almost complete complement of *S. kudriavzevii* chromosomes (Borneman *et al.*, 2012). The genomic composition of AWRI 1116 remains to be determined; however, preliminary data infer an incomplete complement of *S. kudriavzevii* chromosomes (authors' own unpublished data). Our observations are, therefore, in accordance with other studies, where the presence of the *S. kudriavzevii* genome affected aroma

compound production during alcoholic fermentation (Swiegers *et al.*, 2009; Bellon *et al.*, 2011). An unexpected outcome was that all novel hybrids generated in this study produced very low levels of acetic acid during fermentation, a desirable trait in wine production (Ribereau-Gayon *et al.*, 2006).

In conclusion, we demonstrate that wine flavor diversity associated with *Saccharomyces* interspecific hybrids can be combined with desirable oenological traits of *S. cerevisiae* mutants, through application of a mass-mating approach taking advantage of their differential phenotypic traits as selectable markers.

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2.2 OTHER UNPUBLISHED ARTICLES

2.2.1 Interactions between industrial yeasts and contaminants in grape juice affect wine composition profile

Medsebojni vpliv med industrijskimi sevi kvasovk in kontaminanti v grozdnem soku vplivajo na sestavo vina

Etjen Bizaj, Chris Curtin, Neža Čadež and Peter Raspor

Journal of Agricultural and Food Chemistry (2012), Submited for peer review

The interaction between four industrial wine yeast strains and grape juice contaminants during alcoholic fermentation was studied. Industrial strains of *Saccharomyces cerevisiae* (AWRI 0838), *S. cerevisiae* mutant with low H_2S production phenotype (AWRI 1640), interspecies hybrid of *S.cerevisiae* and *Saccharomyces kudriavzevii* (AWRI 1539) and a hybrid of AWRI 1640 and AWRI 1539 (AWRI 1810) were exposed separately to fungicides pyrimethanil (Pyr, 10 mg/L) and fenhexamid (Fhx, 10 mg/L) as well as with the most common toxin produced by molds on grapes ochratoxin A (OTA, 5µg/L). The strains were exposed to the contaminants during alcoholic fermentation of *Vitis vinifera* cv. "Sauvignon Blanc" juice and during extended contact with yeast lees after the completion of alcoholic fermentation.

Yeast's capacity to remove contaminants from media was determined by GC-MS (Pyr), LC-MS/MS (Fhx) and HPLC (OTA) at the end of the alcoholic fermentation, and after extended contact (7 days) with the media. All the strains were able to remove contaminants from media, moreover, after the extended contact; the concentration of contaminants was in most cases even lower.

On the other hand, contaminants were found to strongly impair fermentation performance and metabolic activity for all yeast strains studied. Wine's chemical profile was analyzed by HPLC (ethanol, volatile acidity, concentration of fructose, glucose, glycerol and organic acids) and the aromatic profile was analyzed by using a (GC/MS) stable isotope dilution technique (ethyl esters, acetates and aromatic alcohols) and Kitanawa tubes (H_2S). The chemical composition of wine with added contaminants was in all cases significantly different from the control. Of particular note, the quantity of desired aromatic compounds produced by yeast was significantly lower.

In this study we observed that interactions between industrial yeast strains with different genetic background, and different chemical contaminants; Pyr, Fhx and OTA can influence low quality chemical composition of wine.

Interactions between Industrial Yeasts and Contaminants in Grape Juice Affect Wine Composition Profile

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ABSTRACT

The interaction between four industrial wine yeast strains and grape juice contaminants during alcoholic fermentation was studied. Industrial strains of *Saccharomyces cerevisiae* (AWRI 0838), *S. cerevisiae* mutant with low H_2S production phenotype (AWRI 1640), interspecies hybrid of *S.cerevisiae* and *Saccharomyces kudriavzevii* (AWRI 1539) and a hybrid of AWRI 1640 and AWRI 1539 (AWRI 1810) were exposed separately to fungicides pyrimethanil (Pyr, 10 mg/L) and fenhexamid (Fhx, 10 mg/L) as well as with the most common toxin produced by molds on grapes ochratoxin A (OTA, 5µg/L). The strains were exposed to the contaminants during alcoholic fermentation of *Vitis vinifera* cv. "Sauvignon Blanc" juice, and during the extended contact with yeast lees after completion of alcoholic fermentation.

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In this study we observed that interactions between industrial yeast strains with different genetic background, and different chemical contaminants; Pyr, Fhx and OTA can influence low quality chemical composition of wine.

KEYWORDS: *Saccharomyces sensu stricto*, interspecies hybrids, interaction, pyrimethanil, fenhexamid, ochratoxin A, aromatic profile, fermentation kinetics, H_2S, Sauvignon blanc

INTRODUCTION

The chemical composition of grape juice is mainly a consequence of vine physiological processes.[1] However, grapes in the vineyard may undergo microbiological spoilage by molds, bacteria and yeasts, leading to formation of toxic secondary metabolites; such as mycotoxins.[1-3] On the other hand, the use of pesticides in order to preserve the crop from microbial and other spoilage can itself induce changes in grape physiology and subsequent grape juice chemical composition.[4]

In grape/wine production, *Botrytis cinerea* is a fungal pathogen of serious economical importance. Its infection of grape bunches induces better conditions for growth of other spoilage microbiota.[5] Among these *Aspergillus* and *Penicillium*, which are present on grape berries from early stages of development, are producers of ochratoxin A (OTA), especially *A. carbonarius.*[2, 6] OTA is a dangerous mycotoxin, that can be found in grape juices and wines.[1, 7, 8] Many studies have investigated its removal from grape juice, wine and other media, to reduce its negative impact on human health.[9-25] It was shown[15, 18, 24] that yeasts are able to reduce OTA concentration by its adsorption on the yeast cell wall during alcoholic fermentation; predominantly to mannoproteins which are released from yeast cell walls in the late stages of alcoholic fermentation. However, contrasting data has also been published.[14] This may be due to differences in media omposition and physico-chemical conditions, which have been shown to strongly affect the OTA removal potential of yeast strains.[26, 27]

If the effect of yeasts on the concentration of OTA in the fermenting media has been widely studied, this is not the case for OTA's influence on yeast metabolism in fermenting media. In a previous study we demonstrated that OTA at higher concentrations (~5µg/L) during alcoholic fermentation impaired on yeast fermentative capacities and induced a higher volatile acidity production in synthetic media.[15]

To prevent microbiological spoilage of grapes and formation of mycotoxins such as OTA, many fungicides and other phytopharmaceuticals are used. However, in years when the conditions for *Botrytis* infection are favorable, such control measures may cause maximum permitted residue levels to be exceeded.[4, 28]

During processing of grapes into wine, the concentrations of fungicides are significantly reduced.[29, 30] Besides other types of processing, wine yeasts were found to be able to decrease the concentration of pesticides during, and especially after the end of alcoholic fermentation when mannoproteins are released from yeast cell walls. However the type of fungicide and physico-chemical properties of the media has a strong effect on contaminants removal. [24, 26, 27, 31-33] Pesticides were found to be removed by degradation or by adsorption but the latter is more frequent and effective.[4] Moreover, a simultaneous degradation/ adsorption action of yeasts was also observed.[34]

The effect of pesticides, especially fungicides on grape juice/ wine microbiota has been studied widely. It has been demonstrated that some fungicides are able to affect the ecology of inoculated and spontaneous alcoholic fermentations of grape juice and synthetic media, as well as their kinetics.[33, 35-38] However, most of the studies showed that fungicides of the older generation had higher toxicity in comparison to the more recently developed.[5, 39-42] Besides primary metabolism, fungicides were found to also affect the secondary metabolism of wine yeasts. During alcoholic fermentation, fungicides were found to negatively affect yeast's aromatic compound production, this way negatively affecting wine flavor. [28, 43-48]

In recent years the trend has been to use selected yeasts for the alcoholic fermentation, because these can guarantee the smooth development of the process, avoid the production of off-flavors and generate positive aromas that improve the sensory properties of the wine.[49] In order to improve wine aromatic composition other alternative techniques of inoculation have been

adopted; mixed/sequenced inocula of different strains[50,51], introduction of non-*Saccharomyces* selected yeasts[52,53] and the use of interspecies hybrids of *Saccharomyces* yeasts; i.e. hybrids of *S. cerevisiae* and *S. kudriavzevii*, were found to have very good aromatic production potential.[48,54]

The aim of this study was to uncover the interaction of four genetically different industrial wine yeast strains; *S. cerevisiae* (AWRI 0838), a *S. cerevisiae* mutant with low H_2S production phenotype (AWRI 1640), an interspecies hybrid of *S. cerevisiae* and *S. kudriavzevii* (AWRI 1539) and the hybrid of the last two strains (AWRI 1810) with fungicides pyrimethanil and fenhexamid and the mycotoxin OTA. With determination of the removal potential of strains of such different genomic background and the potential of contaminants to affect yeast metabolism (fermentation kinetics and aromatic compound production) we tried to answer; whether the abusive use of fungicides affects more negatively the final product, than the presence of OTA during alcoholic fermentation.[1,55,56]

MATERIALS AND METHODS

Media and Reagents

The fermentation media was a cv. Sauvignon blanc 2005 (SB05) grape juice: 128.5 g/L reducible sugars, titrable acidity (pH 8.2) 5.1 g/L, pH 3.19, SO_2 (free) 10 mg/L, SO_2 (total) 19 mg/L, YAN 235 mg/L. YPD was composed by 2% D-glucose (Sigma-Aldrich, USA), 1% yeast extract, 1% peptone, and solidified with 2% agar; (all Amyl Media Pty Ltd, Australia). Chemically Defined Must (CDM).[58] All liquid media were sterilized by filtration before use sterilized (0.65/0.22 µm; Sartorius Germany). Ochratoxin A as well as pyrimethanil (*N*-(4,6 dimethylpyrimidin-2-yl)-aniline) and fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide) (all analytical standards) were obtained from Sigma-Aldrich (USA). The stock solutions of the three contaminants were prepared in 1.0 mL of 80 % v/v Et-OH, previously sterilized by filtration (0.22 µm; Sartorius Germany). Et-OH was a pure reagent (Merck, Germany) diluted with deionized H_2O.

Yeast Strains

The yeast strains used in this study were all food-grade industrial wine yeasts; *Saccharomyces cerevisiae* AWRI 0838, *Saccharomyces cerevisiae* AWRI 1640[58] a mutant with low H_2S phenotype, AWRI 1539 an interspecies hybrid of *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* and AWRI 1810[33] the hybrid of AWRI 1640 and AWRI 1539. Three days old cultures on YPD solid plates grown at 28 °C were inoculated into 8 mL sterile YPD broth for 24 h at 28 °C (in 12 ml sterile falcon tubes). All 8 mL were later transferred into 16 mL of sterile CDM[57] for 24 h (into sterile 50 mL falcon tubes). The concentrations of yeast cells for the inocula were counted by hemocytometer.

Fermentation Assays

Alcoholic fermentations were carried out in sterile 250 mL fermentation flasks (Schott, Germany) containing 200 mL of media. Each of four strains was inoculated at the final concentration of $1x10^6$cells/mL (all in triplicate). Four types of media were prepared: [1] Control, which was composed of 2.5L SB05 and 1 mL Et-OH 80 % v/v; [2] Pyr, and [3] Fhx which were composed of 2.5L SB05 and 1 mL Et-OH 80 % v/v containing, respectively, 25.0 mg of pyrimethanil and fenhexamid to reach the final concentration of 10 mg/L[33] ; [4] OTA, composed of 2.5 L of sterile SB05 and 1 mL of sterile Et-OH 80 % v/v containing 12.5 µg of OTA to reach the final concentration of 5.0 µg/L.[15]

The fermentation flasks were equipped with precision gas detector tubes (Kitagawa, Japan) a trap-based method for H_2S quantification during fermentation.
The assays were performed at 17 ºC, with rotary shaking at 150 rpm. The fermentation kinetics was followed by CO_2 weight loss measurement every 24h. The fermentations were defined finished when CO_2 release was lower than 0.1 g/100mL/day and the concentration of reducible sugars was lower than 2 g/L (Clinitest®, Bayer, Germany).
Samples of wine were taken after the end of fermentation for the determination of volatile and non-volatile chemical compounds as well as the concentration of contaminants. The samples were taken from homogenized media in aseptic conditions, centrifuged (5 min at 11200 x g), and the clean supernatant was frozen for analysis.

Extended Yeast Lees – Wine Contact
In order to determine the removal potential of yeast lees for contaminants a 7 day – long extended contact time with daily mixing was performed.[15, 24, 31-33] After the contact period, the samples were taken from homogenized media in aseptic conditions, centrifuged (5 min at 11200 x g), and the clean supernatant was frozen for analysis.

Analysis of the Principal Chemical Compounds in Wine
The fermented SB05 wines were analyzed for glucose, fructose, ethanol, glycerol, acetic, citric, malic and tartaric acid. Their concentration in media was analyzed by HPLC, using a Bio-Rad HPX-87H column as described previously.[58,59]

Analysis of Fermentation Products
Samples of fermented SB05 wines were prepared as follows: from each treatment the same aliquots of the three replicates were taken and mixed together into one sample (from 48 fermentations; 16 final samples). Samples were prepared in 2 dilutions 1/20 and 3/10 with Model Wine (13.8 % ethanol, 10 % potassium hydrogen tartrate, pH adjusted with tartaric acid to 3.5). Samples were prepared and analyzed for ethyl esters, acetates and aromatic alcohols, in a randomized order with a blank run every 10 samples.
The analysis was performed on an Agilent 7890 gas chromatograph equipped with Gerstel MPS2 multi-purpose sampler and coupled to an Agilent 5975C VL mass selective detector. Instrument control and data analysis was performed with Agilent G1701A Revision E.02.00 ChemStation software. The gas chromatograph is fitted with a 30 m x 0.18 mm ResteckStabilwax – DA (crossbondcarbowax polyethylene glycol) 0.18mm film thickness that has a 5m x 0.18 mm retention gap. Helium (Ultra High Purity) is used as the carrier gas with linear velocity 24.6 cm/s, flow-rate 0.78 mL/min in constant flow mode. The oven temperature is started at 33 °C, held at this temperature for 4 min then increased to 60 °C at 4 °C/min, then heated at 8 °C/min to 230 °C and held at this temperature for 5 min. The conditions of large volume headspace sampling used were as follows: the vial and its contents are heated to 40 °C for 10 minutes with agitation (speed 750 rpm, on time 80 s, off time 1 s). A heated (55 °C) 2.5 mL syringe penetrates the septum (27.0 mm) and removes 2.5 mL of headspace (fill speed 200 µL/s). The contents of the syringe are then injected into a Gerstel PVT (CIS 4) inlet fitted with a Tenax TA inlet liner (0.75 mm I.D., pre-conditioned in the GC inlet at 200 °C for 1 hour and then ramped to 350 °C to remove all contaminants before first injection).
The inlet conditions used were as follows:
Prior to injection the inlet is cooled to 0 °C with liquid nitrogen. While maintaining 0 °C the sample is introduced to the inlet at 25.0 µL/s (penetration 22.0 mm) using split mode (split ratio 33:1, split flow 25.78 mL/min). Following capture of analytes on the Tenax liner the injector is heated to 330 °C at 12 °C/min (pressure 24.6).

The mass spectrometer conditions used were as follows:
The mass spectrometer quadrupole temperature is set at 150 °C, the source was set at 250 °C and the transfer line is held at 280 °C. Positive ion electron impact spectra at 70 eV are recorded in selective ion monitoring (SIM) mode and Scan mode simultaneously (relative EM volts) with a solvent delay of 4.0 min. All data processing was performed on Agilent G1701A Revision E.02.00 ChemStation software.

Determination of Fungicide Residues
The extraction procedure and determination of pyrimethanil and fenhexamid residues in fermented SB05 wines was done using a gas chromatography–mass spectrometry system (GC–MS) for pyrimethanil and liquid chromatography- tandem mass spectrometry system (LC/MS/MS) for fenhexamid according to methods previously described.[29, 60]
For the determination of pyrimethanil, in the GC-MS analysis we used HP-5MS capillary column (30 m, 0.25 mm ID, 0.25 lm film). Injector temperature was 250 °C, ion source temperature was 230 °C, auxiliary temperature was 280 °C and quadrupole temperature was 150 °C. GC oven temperature was programmed from 55 °C (held for 2 min) to 130 °C at rate 25 °C/min (held for 1 min) then to 180 °C at rate 5 °C/min (held for 30 min), then to 230 °C at rate 20 °C/min (held for 16 min), then to 250 °C at rate 20 °C/min (held for 13 min), then to 280 °C at rate 20 °C/min (held for 20 min). The helium constant flow was 1.2 ml/min. The liner used was Agilent 5181-3316.
The content of fenhexamid residues in methanol extract was analyzed using a liquid chromatography (PE200, Perkin Elmer, Waltham, Massachusetts, USA) coupled with triple quadrupole mass detector (3200 QTrap, Applied Biosystems MDS Sciex, Concorde, Canada). Turbo spray temperature was kept at 650 °C. The compounds were separated on a Gemini C18 column, 250 mm x 4 mm (Phenomenex, Torrance California, USA). Gradient elution was used for pesticide separation. Mobile phase A consisted of 75 % 5 mM $HCOONH_4$ and 25 % methanol (v/v) with 0.1 % formic acid added and mobile phase B of 5 % 5 mM $HCOONH_4$ and 95 % methanol (v/v) with 0.1 % formic acid added. The initial conditions (100 % mobile phase A) were maintained for 5 min, then linear gradient was applied and, in 30 min, 100 % of mobile phase B was reached and maintained for 15 min. Conditioning of column to initial mobile phase A was carried out for 10 min. Data was collected in multi reaction monitoring (MRM) mode (dwell time 5 ms), for each compound, two MRM transitions were monitored.

Determination of Ochratoxin A Residues
The fermented SB05 wines and samples of wines that have been collected after the extended contact phase with yeast lees were analyzed OTA. Their concentration in media was analyzed by means of immunoaffinity column clean-up and HPLC, as described previously.[61]

Statistical Analysis
Statistical analysis were done by using GLM (General Linear Model).
Statistical model: $y_{ijk} = \mu + T_i + S_j + T*S_{ij} + e_{ijk}$; y_{ijk} = controled value, μ = average value, T_i = effect of i-treatment; i=1-8, S_j = effect of j-strain; j=1-4, $T*S_{ij}$ = effect of interaction between i-treatment and j-strain, e_{ijk} = rest. (SAS Software Version 8.01. 1999. Cary, SAS Institute Inc.).
The data of fermentation kinetics were statistically analysed by intervals of standard deviation (Microsoft Office Excel 2003, USA). The statistical level of significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Fermentation Kinetics

The fermentation kinetics at 17 °C in the SB05 grape juices varied for the genetically different yeast strains; the fastest fermentation kinetics in control treatments was observed for AWRI 1539 in 7 days; AWRI 0838 and 1810 were intermediate, finishing the fermentation in 9 days, while AWRI 1640 displayed the slowest fermentation rate, completing fermentation in 12 days (Figures 1-4). Differences between genetically different strains were expected in control media.[15,33,48,62] More interestingly, all strains responded in similar fashion when challenged with contaminants, with significantly slower fermentation rates.Similar trend can be observed for all strains; no significant differences were observed in the spiked media in the lag and logarithmic phase. However in Fhx fermentations, the stationary phases were reached earlier for all strains, showing that Fhx was less inhibitory if compared with OTA and Pyr. In previous works[41,63], no negative effect of fenhexamid was found on wine yeast fermentation kinetics, even at concentrations greater than that permitted by legislation (3 mg/L), and not even for its UV light degradation product (7-chloro-6-hydroxy-2-(1 methylcyclohexyl)-1,3-benzoxazole (CHB).[65] Recently Bizaj and colleagues[33] demonstrated fenhexamide's negative effect on kinetics at higher concentrations, that is 10mg/L, during wine yeasts fermentations in synthetic media.[33] Here in the present paper we confirmed that Fhx can negatively affect genetically different wine yeasts even in grape juice.The negative effect of Pyr on alcoholic fermentation of wine yeasts was observed previously.[33] Its toxicity was shown to be greater than the toxicity of Fhx in synthetic media.[33] Previously published data on Pyr toxicity in synthetic media was confirmed also in grape juice. The effect of Pyr was most prominent for AWRI 1539. Its fermentation rate was initially the fastest, but after the 3rd day of fermentation a drastic decrease in fermentation rate can be seen. Similar trend was observed also by Bizaj and colleagues[33]; where strains exhibiting faster fermentation rates were more affected by the toxicity of contaminants. Moreover, this trend can be strengthened by pyrimethanil's lower negative effect on slower fermenting strains; AWRI 1810, 0838 and 1640. OTA was found to affect wine yeasts during fermentation in synthetic media for the first time by Bizaj and colleagues.[15] In the present study we demonstrated that OTA had effect on fermentation kinetics for different wine yeasts in grape juice as well. Moreover, in concentration addedin the media its toxicity seemed to be similar (AWRI 1539, 1640) or identical to pyrimethanil for fermentations of AWRI 1810 and 0838 in its presence.In assays with OTA and Pyr, all the fermentations tended to be sluggish or stuck, producing less CO_2 in comparison to the control and Fhx fermentations, suggesting their more intensive negative effect on all wine yeast strains studied.

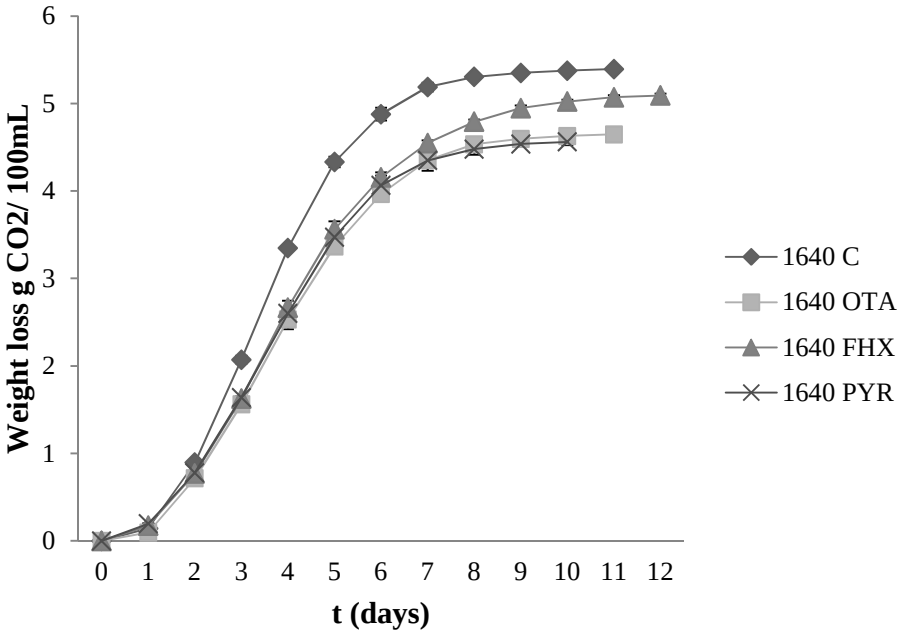

Figure 1. Fermentation kinetics of fermentation assays with the strain AWRI 1640

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L)
*all concentrations are refered to initial concentration of contaminants in grape juice
*data reported are mean values and standard deviations of three independent experiments carried out in identical conditions

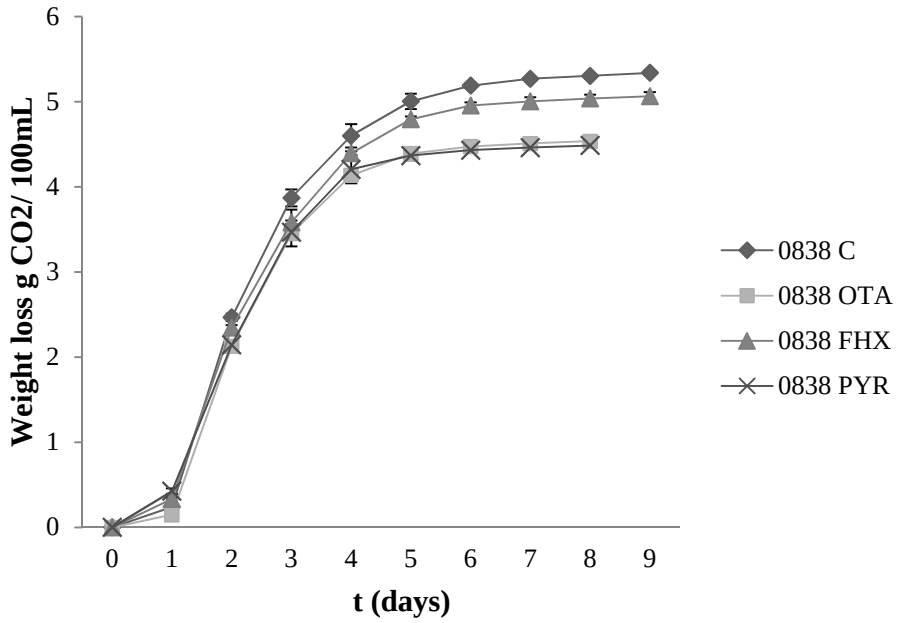

Figure 2. Fermentation kinetics of fermentation assays with the strain AWRI 0838

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L)
*all concentrations are refered to initial concentration of contaminants in grape juice
*data reported are mean values and standard deviations of three independent experiments carried out in identical conditions

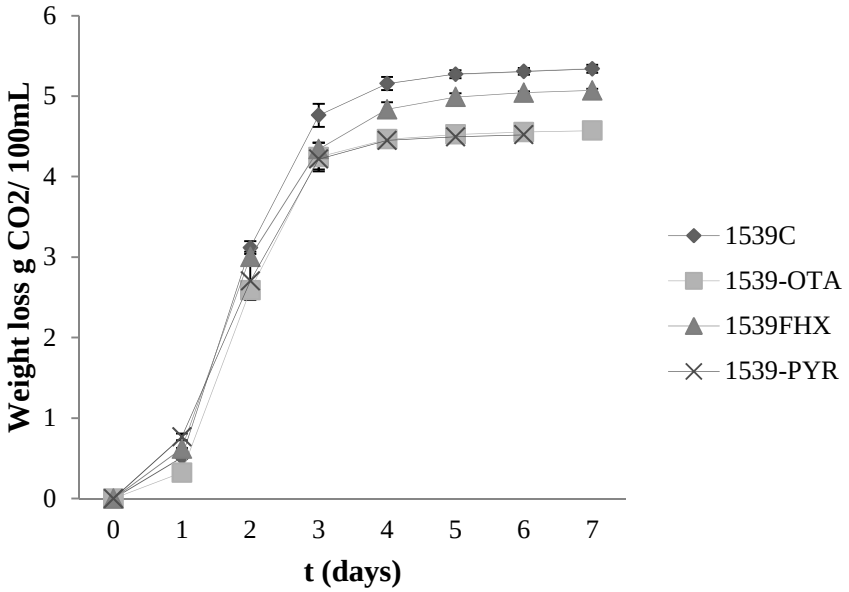

Figure 3. Fermentation kinetics of fermentation assays with the strain AWRI 1539

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L)
*all concentrations are refered to initial concentration of contaminants in grape juice
*data reported are mean values and standard deviations of three independent experiments carried out in identical conditions

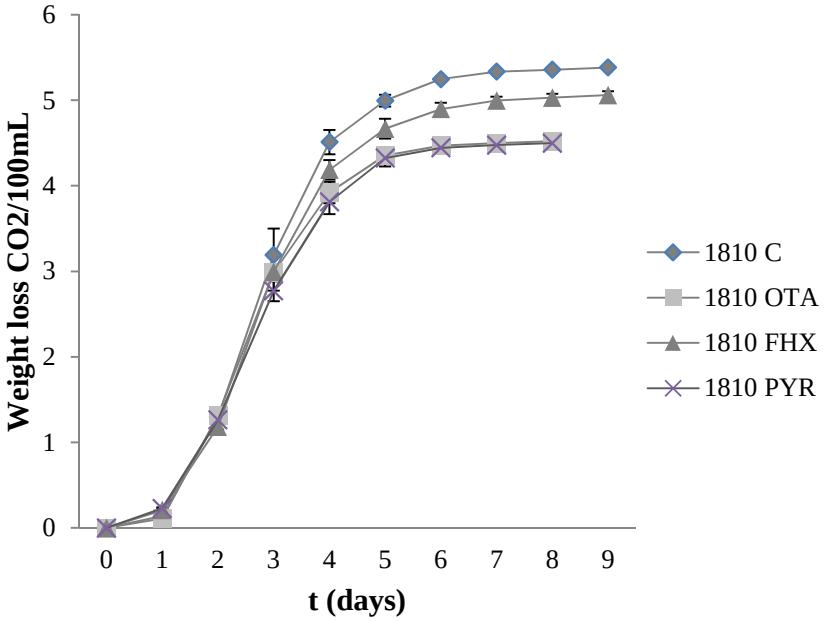

Figure 4. Fermentation kinetics of fermentation assays with the strain AWRI 1810

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L)
*all concentrations are refered to initial concentration of contaminants in grape juice
*data reported are mean values and standard deviations of three independent experiments carried out in identical conditions

Off-Flavor Production During Alcoholic Fermentation: H_2S

H_2S is a natural product of yeast metabolism during alcoholic fermentation. The majority of H_2S produced during winemaking occurs as a result of the biosynthesis of sulphur -containing amino acids methionine and cysteine. This occurs in low concentrations in grape juice, through the *sulfate reduction sequence* (SRS) and causes an off-flavour that reminds of rotten eggs.[58] Many genetic engineering strategies have been used for limiting H_2S production by yeast[65-67]. Cordente and colleagues[58] produced the first food grade low H_2S producing strains; among them AWRI 1640 which has a point mutation in the *MET10* gene (G176A), responsible for its very low H_2S production phenotype. All AWRI 1640 fermentations performed in this study were free of detectable H_2S according to precision gas detector tubes (Kitagawa, Japan). The three other strains had different capacities for H_2S production; AWRI 1539 was previously shown to be a relatively high H_2S producer[48], while AWRI 0838 and AWRI 1810, the hybrid between AWRI 1539 and AWRI 1640[48], are intermediate producers. From Table 1 can be seen that the H_2S production phenotypes were confirmed for all the strains in control assay. However, the contaminants seem to have different effects on their H_2S production during fermentation. The strain AWRI 0838 produced the highest amount of H_2S in control fermentations, suggesting that all contaminants impaired H_2S production. On the other hand, for AWRI 1810 Fhx and Pyr stimulated H_2S productions, since in OTA and control assays the amount of produced H_2S was significantly lower. The highest H_2S producer, AWRI 1539, was affected similarly as in the case of fermentation kinetics; that is when challenged with Pyr and OTA AWRI 1539 produced lower amounts of H_2S than with Fhx and the control. These results suggest that there were interactions between the contaminants and yeast strains, with regard to H_2S formation. Fenhexamid, pirymethanil and ochratoxin A, were previously found to have effects on metabolic pathways during alcoholic fermentations[15, 33, 37, 38], however here we demonstrate that all of them have the potential to affect H_2S production pathway during alcoholic fermentation in grape juice.

Table 1. Concentration of H_2S produced during alcoholic fermentation (mg/L) of sterile filtered cv. "Sauvignon Blanc" 2005 grape juice (mg/L) by strains AWRI 1640, 0838, 1539, 1810 in assays with contaminants fenhexamid, pyrimethanil and ochratoxin A. The assays were conducted at 17 °C. Data reported are mean values and standard deviations of three independent experiments carried out under identical conditions.

Strain/Treatment	Control	OTA	Fhx	Pyr
1640	0 ± 0^{aD}	0 ± 0^{aC}	0 ± 0^{aD}	0 ± 0^{aD}
0838	19 ± 1^{aB}	10^{bBn}	6^{bCn}	6^{bCn}
1539	365^{aAn}	245^{bAn}	347 ± 15^{aA}	153 ± 6^{cA}
1810	13^{aCn}	11^{aBn}	20 ± 1^{bB}	17^{abBn}

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L)
*all concentrations are referred to initial concentration of contaminants in grape juice
*a, b, c, d; significant difference at $P \leq 0.05$ (mean values with the same letter in the same line do not differ significantly; effect of treatment)
*A, B, C, D; significant difference at $P \leq 0.05$ (mean values with the same letter in the same column do not differ significantly; effect of strain)
*n=number of replicates is 2

The Principal Chemical Compounds in Wine

The principal chemical compounds analyzed at the end of fermentation were citric, tartaric, malic, succinic, and lactic acids, glycerol, acetic acid, ethanol, glucose and fructose (Table 2). These are considered to be the basic technological data after the grape juice is fermented into wine; and are dependent on the basic composition of grape juice and the yeast metabolism.[1]
SB05 grape juice had an initial concentration of reducible sugars of 128.5 g/L; at this concentration and with 235 mg/L of YAN, all the strains in all four types of assays were able to complete fermentation, since no residual sugar in media was found after the end of alcoholic fermentation.
Malic and citric acids are naturally present in grape juice, and these are intermediate products in primary yeast metabolism as well.[1, 68, 69] In our study the highest concentration of malic acid was found in all assays when AWRI 1640 was used. Moreover no detectable differences between the four treatments were found for this strain, suggesting that since this strain was produced by non-specific method of chemical mutagenesis,[58] malic degradation enzyme might have been affected.[70,71] Interestingly, higher production of malic acid than AWRI 0838 and 1539 was characteristic also for a triple hybrid AWRI 1810 which was showing intermediate phenotype, suggesting that has inherited a part of AWRI 1640 genome background. Except for the strain AWRI 1640; all strains degraded more malic acid if a contaminant was present in the media. Similar trend was observed also for citric acid in terms of contaminant effect; on the other hand, there were not present any particular trends in-between different strains. Succinic acid is considered to be particularly important for sensory wine quality.[1] The production of succinic acid was significantly lower in all assays where the contaminants were present, meaning that their presence in media negatively affected its production. Strain dependency was evident again; the inheritance previously observed for malic acid degradation was present for succinic acid production as well. The highest production was observed for AWRI 0838 and 1640, the lowest for AWRI 1539 and intermediate for AWRI 1810. Strain dependency was alsoobserved to be more important in the case of lactic acid and acetic acid production. AWRI 0838 was the highest producer of lactic acid followed by AWRI 1810, 1539 and 1640 in control assays; on the other hand there is not present a particular trend of contaminant affection of its production, except for AWRI 1640, where contaminants negatively affected lactic acid production. AWRI 1539 has a tendency to produce higher levels of acetic acid [48], which can be seen also in this study; but none of the contaminants affected acetic acid production. Similarly to lactic acid, AWRI 1640 was strongly affected by the presence of contaminants, suggesting that metabolic pathways that succeeded the synthesis of pyruvate are particularly susceptible to contaminants in this strain.
Glycerol production during alcoholic fermentation was affected when contaminants were present in media. AWRI 1640 and 1539 were clearly impaired for glycerol production by all three contaminants, especially the latter in the pyrimethaniltreatment. On the other hand AWRI 0838 and 1810 were less sensitive overall, moreover pyrimethanil stimulated their glycerol production. The production of ethanol was the most negatively affected. Even though all the reducible sugars were consumed by yeasts during fermentation, it seems that due to contaminant action on metabolic pathway rising concentration of ethanol in media, yeasts were not able to convert it to ethanol. Yeasts exposed to contaminants produced roughly 14% lower concentrations of ethanol. Again, pyrimethanil was found to be the most toxic during fermentation. Its effect was the most negative for AWRI 1539, where a 35.8 % decrease in ethanol production was observed. However, this was expected from the fermentation kinetics observed (Figure 3). Similar data were found for AWRI 1640 where pyrimethanil was found to

be the most toxic. On the other hand, we could not observe any significant differences between assays with contaminants for AWRI 0838 and 1640.

Table 2. Concentration of the principal chemical compounds in finished wines of sterile filtered cv. "Sauvignon Blanc" 2005 grape juice (g/L) by strains AWRI 1640, 0838, 1539, 1810 with contaminants fenhexamid, pyrimethanil and ochratoxin A. The assays were conducted at 17 °C. Data reported are mean values and standard deviations of three independent experiments carried out under identical conditions.

Compound	Treatment	Strain				
		1640	0838	1539	1810	
citric acid	Control	0.181 ± 0.009^{bA}	0.210 ± 0.007^{aA}	0.212 ± 0.013^{aA}	0.195 ± 0.006^{abA}	$p_t < 0.001$
	OTA	0.167 ± 0.017^{bA}	0.190 ± 0.004^{aB}	0.182 ± 0.004^{abB}	0.171 ± 0.006^{bB}	$p_s < 0.001$
	Fhx	0.182 ± 0.013^{aA}	0.211 ± 0.006^{bA}	0.204 ± 0.002^{aAB}	0.197 ± 0.003^{aA}	$p_{t*s} < 0.001$
	Pyr	0.163 ± 0.010^{bA}	0.197 ± 0.002^{aAB}	0.146 ± 0.009^{cC}	0.185 ± 0.005^{aA}	
tartaric acid	Control	2.039 ± 0.062^{aA}	2.088 ± 0.037^{aA}	1.855 ± 0.212^{aB}	1.938 ± 0.135^{aA}	$p_t < 0.001$
	OTA	1.728 ± 0.148^{bB}	1.919 ± 0.076^{aB}	1.993 ± 0.063^{aAB}	1.640 ± 0.041^{bC}	$p_s < 0.0850$
	Fhx	1.960 ± 0.047^{bA}	1.758 ± 0.072^{cB}	2.065 ± 0.028^{aA}	1.730 ± 0.056^{cB}	$p_{t*s} < 0.001$
	Pyr	$1.735\pm a0.097^{bB}$	1.874 ± 0.099^{aB}	1.555 ± 0.097^{bC}	1.859 ± 0.138^{aB}	
malic acid	Control	3.059 ± 0.028^{aA}	2.349 ± 0.008^{cA}	2.337 ± 0.010^{cA}	2.553 ± 0.063^{bA}	$p_t < 0.001$
	OTA	2.900 ± 0.164^{aA}	2.156 ± 0.024^{cB}	2.221 ± 0.089^{bcAB}	2.377 ± 0.061^{bB}	$p_s < 0.001$
	Fhx	3.006 ± 0.065^{aA}	2.164 ± 0.017^{cB}	2.197 ± 0.019^{cB}	2.381 ± 0.020^{bB}	$p_{t*s} < 0.001$
	Pyr	3.077 ± 0.142^{aA}	2.146 ± 0.056^{cB}	1.748 ± 0.112^{dC}	2.459 ± 0.040^{bAB}	
succinic acid	Control	1.137 ± 0.029^{aA}	1.113 ± 0.021^{aA}	0.929 ± 0.034^{cA}	1.046 ± 0.017^{bA}	$p_t < 0.001$
	OTA	$0{,}962\pm0.056^{abB}$	0.998 ± 0.011^{aB}	0.791 ± 0.017^{cB}	0.929 ± 0.026^{bB}	$p_s < 0.001$
	Fhx	1.028 ± 0.039^{aB}	0.981 ± 0.004^{bC}	0.821 ± 0.007^{cB}	0.950 ± 0.012^{bB}	$p_{t*s} < 0.001$
	Pyr	0.991 ± 0.055^{bB}	1.057 ± 0.010^{aAB}	0.661 ± 0.027^{cC}	1.005 ± 0.014^{abAB}	
lactic acid	Control	0.059 ± 0.003^{dA}	0.103 ± 0.004^{aA}	0.081 ± 0.006^{cA}	0.091 ± 0.002^{bA}	$p_t < 0.001$
	OTA	0.043 ± 0.005^{bBC}	0.113 ± 0.046^{aA}	0.063 ± 0.009^{bAB}	0.089 ± 0.011^{abA}	$p_s < 0.001$
	Fhx	0.049 ± 0.008^{cB}	0.096 ± 0.004^{aA}	0.080 ± 0.004^{bA}	0.091 ± 0.000^{aA}	$p_{t*s} < 0.0097$
	Pyr	0.037 ± 0.003^{cC}	0.069 ± 0.002^{aB}	0.049 ± 0.005^{bB}	0.075 ± 0.007^{aA}	
glycerol	Control	3.596 ± 0.118^{cA}	3.940 ± 0.071^{bA}	4.189 ± 0.084^{aA}	3.519 ± 0.042^{cAB}	$p_t < 0.001$
	OTA	3.044 ± 0.223^{cB}	3.700 ± 0.078^{aAB}	3.759 ± 0.058^{aB}	3.338 ± 0.062^{bB}	$p_s < 0.001$
	Fhx	3.116 ± 0.115^{dB}	3.664 ± 0.022^{bB}	3.837 ± 0.064^{aB}	3.489 ± 0.044^{cAB}	$p_{t*s} < 0.001$
	Pyr	3.176 ± 0.216^{cB}	4.095 ± 0.015^{aA}	3.168 ± 0.112^{cC}	3.706 ± 0.059^{bA}	
acetic acid	Control	0.044 ± 0.006^{bA}	0.024 ± 0.006^{bA}	0.120 ± 0.022^{aA}	0.022 ± 0.011^{bA}	$p_t < 0.001$
	OTA	0.026 ± 0.004^{bB}	0.024 ± 0.006^{bA}	0.135 ± 0.013^{aA}	0.025 ± 0.006^{bA}	$p_s < 0.001$
	Fhx	$0{,}026\pm0.007^{bB}$	$0{,}018\pm0.001^{bA}$	0.133 ± 0.013^{aA}	0.027 ± 0.003^{bA}	$p_{t*s} < 0.001$
	Pyr	0.006 ± 0.000^{cC}	0.029 ± 0.010^{bA}	0.095 ± 0.006^{aA}	0.030 ± 0.008^{bA}	

Compound	Treatment	Strain				
		1640	0838	1539	1810	
ethanol	Control	66.523±0.732^{aA}	65.515±0.935^{aA}	65.599±2.337^{aA}	65.298±1.624^{aA}	$p_t < 0.001$
	OTA	55.965±2.916^{aB}	56.025±0.076^{aB}	56.584±0.529^{aC}	56.251±0.607^{aB}	$p_s < 0.0025$
	Fhx	59.077±1.140^{aB}	58.784±0.714^{aB}	59.696±1.029^{aB}	59.255±0.589^{aB}	$p_{t*s} < 0.001$
	Pyr	50.983±2.576^{bC}	54.972±0.496^{aB}	42.094±1.024^{cD}	55,384±0.275^{aB}	

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L)
*all concentrations are referred to initial concentration of contaminants in grape juice
*a, b, c, d; significant difference at $P \leq 0.05$ (mean values with the same letter in the same line do not differ significantly; effect of strain)
*A, B, C, D; significant difference at $P \leq 0.05$ (mean values with the same letter in the same column do not differ significantly; effect of treatment)

Volatile Fermentation Products in Wine

In Table 3 the production potential of aromatic compounds produced by genetically different yeast strains in fermentative assays with and without contaminants is presented.

The principal esters during alcoholic fermentation are formed by yeasts through enzymatic formation between free alcohols and the acyl-S-CoA[72]. Their concentration in wine is affected by many environmental factors; chemical composition of grape juice[1,44,73,74], fermentation conditions[75, 76] and the latter aging.[77, 78]

Ethyl Esters. Ethyl acetate was the highest produced ethyl ester for all strains. This compound imparts estery and "nail polish" aromas in high concentrations >1600000 µg/L[1, 54], on the other hand; at lower measures < 80000 µg/L it contributes to the pleasant smell of wine; which is the case for all our assays.[1] Its production was impaired in all assays with contaminants; especially when pyrimethanil was present (AWRI 1640, 1539). Strain related sensitivity can be seen as well. Interestingly in the case of AWRI 0838, an *S. cerevisiae* strain; its ethyl acetate production was negatively affected by all contaminants at a similar degree, suggesting high sensitivity of the strain to the three chemical compounds. This is in contrast to the studies of Garcia and colleagues[43], where the non-hybrid *S. cerevisiae* strain was found to be the most resistant to pesticides, including pyrimethanil.

Ethyl hexanoate is well known for its important and positive effect, especially on young wines aroma.[79] In all our treatments, the presence of contaminants in media negatively affected its production, which is in accordance to results obtained by Garcia and colleagues[44]. Moreover, the final concentration in media was shown to be strain dependent.

Ethyl propanoate and ethyl butanoate were all produced below the perception threshold.[80] Ethyl butanoate production was in all treatments negatively affected by contaminants, and showed a strong strain dependency in the production potential. Interestingly, strain dependency in production of ethyl propanoate was present as well, in all assays contaminants stimulated its production, the exception was AWRI 1810.

Ethyl 2-methylpropanoate, ethyl 2-methylbutanoate and ethyl 3-methylbutanoate are derivates of acids considered as indicators of lower quality wine.[81,82] AWRI 0838 and 1810 were the highest producers of ethyl 2-methylpropanoate. Interestingly contaminants positively affected its production, especially Pyr (AWRI 1640 and 0838); and OTA (AWRI 1539 and 1810), which may have the effect of lowering the quality of wines. The second, ethyl 2-methylbutanoate could have been barley detected, moreover ethyl 3-methylbutanoate could not be detected at all in all assays.

Acetates. 2-phenyl ethyl acetate, which confers to young wines the flavor of roses and violets, was found to be below the perception thresholds in all our assays.[44] AWRI 1539 followed by AWRI 1810 were high producers. On the other hand, the two *S. cerevisiae* strains were lower

producers according to Garcia and colleagues.[44] When contaminants were added the production was significantly lower in most of the cases, especially when pyrimethanil was added to assays with AWRI 0838, where none of it was detected. Interestingly, in fermentations with AWRI 1640 in presence of inhibitors, no 2-phenyl ethyl acetate was detected.

The concentration of hexyl acetate detected (cherries, pears) was below the perception threshold [44] in all treatments. Strain dependent production can be observed; where from the highest to the lowest producer were as follows; AWRI 1810, 1539, 0838 and 1640. In all assays where contaminants were added, these had a negative effect on the hexyl acetate production, the only exception was OTA in the case of AWRI 1539.

3-methyl butyl acetate, 2-methyl butyl acetate and 2-methyl propyl acetate are very important for young wine flavor; conferring banana, fruity flavors in white wines.[44,54] As expected, the 3-methyl butyl acetate was produced in the highest concentrations, and a similar trend of strain dependency for production of all three was observed. In all assays where contaminants were present their negative effect can be observed, especially that of pyrimethanil. This is opposite to what was observed by Garcia and colleagues, where its production was stimulated[44], however a different yeast strain was used in their study. The other two acetate esters were produced in far lower concentrations, both being under their perception threshold.[44] The presence of contaminants generally had a negative effect on the concentration of these two esters present.

Alcohols. Higher alcohols are compounds that are produced by yeasts during alcoholic fermentation, from their precursors; amino acids and sugars.[4,80,83,84] Their concentration above certain levels; > 300000µg/L cause unpleasant odor, however at medium concentrations are said to positively affect wine flavor.[1]

Two isoamylic alcohols were analyzed; 2-methyl butanol and 3-methyl butanol, which are considered to be among the major volatiles that confer intensity of fruity flavor to wine.[43,85] Their perception threshold of 7000 µg/L was exceeded in all treatments and their concentration was highly dependent on yeast strain; with interspecies hybrids being higher producers. The presence of contaminants in media affected the concentration of produced amyl alcohols; the concentration of 3-methyl butanol was negatively affected in all cases, on the other hand 2-methyl butanol production by yeasts AWRI 0838 and 1810 was positively affected by Pyr and Fhx, but negatively in case of OTA The same trend of results was observed for butanol as well.

2-methyl propanol concentration in wine depends on alterations in the biosynthesis of the amino acid valine.[86] Its concentration was negatively affected in all our assays with contaminants.

Hexanol is a higher alcohol that is coming from the C6 group, which confers to wines herbaceous flavor.[85,87] Hexanol's perception threshold in wine of 6200 µg/L[80], was not reached in our assays. The highest producer was AWRI 1640; and its, hexanol production was negatively affected only when pyrimethanil was present in the media. For all the other strains, when a contaminant was present reductions of hexanol concentration were observed.

Table 3. Concentration of the fermentation products in finished wines of sterile filtered cv. "Sauvignon Blanc" 2005 grape juice (mg/L) by strains AWRI 1640, 0838, 1539, 1810 with contaminants fenhexamid, pyrimethanil and ochratoxin A. The assays were conducted at 17 °C. Data reported are mean values and standard deviations of three independent experiments carried out under identical conditions.

Compound	Treatment	Strain				
		1640	838	1539	1810	
ethyl acetate	Control	28871 ± 1216^{aA}	20292 ± 854^{cA}	23451 ± 987^{bA}	27464 ± 1156^{aA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	21213 ± 893^{aC}	15247 ± 642^{bB}	21936 ± 924^{aA}	13436 ± 566^{cC}	
	Fhx	24078 ± 1014^{aB}	15292 ± 644^{cB}	16804 ± 708^{cB}	21142 ± 890^{bB}	
	Pyr	14405 ± 607^{aD}	14451 ± 608^{aB}	13218 ± 557^{bC}	13549 ± 571^{abC}	
ethyl propanoate	Control	294 ± 12^{aB}	117 ± 5^{cB}	61 ± 2^{dC}	216 ± 9^{bA}	$p_T<0.0001$ $pS<0.0001$ $p_{T*S}<0.0001$
	OTA	302 ± 12^{aB}	117 ± 5^{bB}	63 ± 3^{cC}	45 ± 2^{dD}	
	Fhx	248 ± 10^{aC}	99 ± 4^{cC}	118 ± 5^{bA}	66 ± 3^{dC}	
	Pyr	334 ± 13^{aA}	129 ± 5^{cA}	107 ± 4^{dB}	151 ± 6^{bB}	
ethyl 2-methylpropanoate	Control	15 ± 1^{cB}	20 ± 1^{bB}	30 ± 2^{aB}	17 ± 1^{cC}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	15 ± 1^{dB}	21 ± 1^{cB}	34 ± 2^{aA}	28 ± 2^{bA}	
	Fhx	14 ± 1^{cB}	16 ± 1^{bC}	13 ± 1^{cC}	25 ± 1^{aB}	
	Pyr	18 ± 1^{bA}	26 ± 1^{aA}	14 ± 1^{cC}	17 ± 1^{bC}	
ethyl butanoate	Control	69 ± 4^{cA}	179 ± 11^{aA}	132 ± 8^{bA}	142 ± 9^{bA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	61 ± 4^{dB}	145 ± 9^{aB}	124 ± 7^{bA}	74 ± 4^{cC}	
	Fhx	59 ± 4^{dB}	133 ± 8^{aB}	88 ± 5^{cB}	117 ± 7^{bB}	
	Pyr	43 ± 3^{cC}	139 ± 8^{aB}	80 ± 5^{bB}	81 ± 5^{bC}	
ethyl 2-methylbutanoate	Control	*	5 ± 0^{aA}	5 ± 0^{aA}	*	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	*	5 ± 0^{aA}	5 ± 0^{aA}	5 ± 0^{a}	
	Fhx	*	5 ± 0^{A}	*	*	
	Pyr	*	*	*	*	
ethyl 3-methylbutanoate	Control	*	*	*	*	$p_T>1.000$ $p_S>1.000$ $p_{T*S}>1.000$
	OTA	*	*	*	*	
	Fhx	*	*	*	*	
	Pyr	*	*	*	*	
ethyl hexanoate	Control	209 ± 13^{cA}	420 ± 25^{aA}	317 ± 19^{bA}	420 ± 25^{aA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	197 ± 12^{cAB}	361 ± 22^{aB}	311 ± 19^{bA}	193 ± 12^{cD}	
	Fhx	181 ± 11^{cB}	343 ± 21^{aB}	268 ± 16^{bB}	283 ± 17^{bB}	
	Pyr	154 ± 9^{cC}	327 ± 20^{aB}	236 ± 14^{bB}	249 ± 15^{bC}	
2-methylpropyl acetate	Control	19 ± 1^{dA}	31 ± 2^{cA}	60 ± 3^{aA}	41 ± 2^{bB}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	17 ± 1^{dB}	26 ± 1^{cB}	57 ± 3^{aA}	37 ± 2^{bB}	
	Fhx	15 ± 1^{cC}	27 ± 1^{bB}	30 ± 2^{bB}	56 ± 3^{aA}	
	Pyr	14 ± 1^{bC}	26 ± 1^{aB}	25 ± 1^{aC}	27 ± 1^{aC}	

2-methylbutyl acetate	Control	32 ± 1^{dA}	60 ± 3^{cA}	71 ± 3^{aA}	65 ± 3^{bA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	29 ± 1^{dB}	44 ± 2^{cC}	55 ± 2^{aB}	49 ± 2^{bB}	
	Fhx	30 ± 1^{cAB}	53 ± 2^{bB}	54 ± 2^{bB}	68 ± 3^{aA}	
	Pyr	25 ± 1^{cC}	42 ± 2^{bC}	*	45 ± 2^{aB}	
3-methylbutyl acetate	Control	195 ± 11^{cA}	838 ± 47^{bA}	833 ± 47^{bA}	1013 ± 57^{aA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	135 ± 8^{dB}	537 ± 30^{bBC}	651 ± 37^{aB}	425 ± 24^{cC}	
	FHX	109 ± 6^{cC}	588 ± 33^{bB}	540 ± 31^{bC}	769 ± 44^{aB}	
	PYR	59 ± 3^{cD}	513 ± 29^{aC}	417 ± 24^{bD}	393 ± 22^{bC}	
2-phenylethyl acetate	Control	*	376 ± 27^{bA}	603 ± 43^{aA}	591 ± 42^{aA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	*	315 ± 22^{cB}	542 ± 29^{aA}	468 ± 33^{bB}	
	Fhx	*	314 ± 22^{cB}	384 ± 27^{bB}	612 ± 44^{aA}	
	Pyr	*	*	323 ± 23^{aB}	303 ± 22^{aC}	
hexyl acetate	Control	29 ± 2^{dA}	78 ± 5^{cA}	99 ± 6^{bA}	125 ± 7^{aA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	22 ± 1^{dB}	53 ± 3^{cC}	93 ± 5^{aA}	67 ± 4^{bC}	
	Fhx	19 ± 1^{dC}	62 ± 4^{cB}	74 ± 4^{bB}	83 ± 5^{aB}	
	Pyr	16 ± 1^{cD}	52 ± 3^{bC}	63 ± 4^{aC}	65 ± 4^{aC}	
2-methyl propanol	Control	451 ± 13^{bA}	471 ± 14^{abA}	421 ± 12^{cA}	494 ± 14^{aA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	434 ± 13^{aA}	443 ± 13^{aB}	402 ± 13^{bB}	388 ± 111^{bD}	
	Fhx	440 ± 13^{bA}	472 ± 14^{aA}	460 ± 12^{abB}	460 ± 13^{abB}	
	Pyr	392 ± 11^{cB}	455 ± 13^{aAB}	417 ± 13^{bB}	420 ± 12^{bC}	
butanol	Control	15476 ± 427^{dA}	19637 ± 542^{bB}	21647 ± 597^{aA}	16972 ± 468^{cB}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	13967 ± 385^{cB}	18798 ± 519^{aB}	19100 ± 527^{aB}	16423 ± 453^{bB}	
	Fhx	14367 ± 396^{cB}	17045 ± 470^{bC}	14515 ± 400^{cC}	20871 ± 576^{aA}	
	Pyr	10396 ± 287^{cC}	20798 ± 574^{aA}	14209 ± 392^{bC}	14996 ± 414^{bC}	
2-methylbutanol	Control	44961 ± 1630^{dA}	55416 ± 2009^{bAB}	61366 ± 2224^{aA}	48855 ± 1771^{cB}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	38196 ± 1385^{cB}	52898 ± 1917^{aB}	52762 ± 1912^{aB}	44958 ± 1630^{bC}	
	Fhx	40593 ± 1471^{cB}	47583 ± 1725^{bC}	40825 ± 1480^{cC}	60208 ± 2182^{aA}	
	Pyr	27140 ± 984^{cC}	58008 ± 2103^{aA}	39144 ± 1419^{bC}	39744 ± 1441^{bD}	
3-methylbutanol	Control	209 ± 6^{cA}	420 ± 12^{aA}	317 ± 9^{bA}	420 ± 12^{aA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	197 ± 6^{cB}	361 ± 11^{aB}	311 ± 9^{bA}	193 ± 6^{cD}	
	Fhx	181 ± 5^{cC}	343 ± 10^{aBC}	268 ± 8^{bB}	283 ± 8^{bB}	
	Pyr	154 ± 5^{cD}	327 ± 10^{aC}	236 ± 7^{bC}	249 ± 7^{bC}	
hexanol	Control	1796 ± 56^{aA}	1599 ± 49^{bcA}	1525 ± 47^{cA}	1681 ± 52^{bA}	$p_T<0.0001$ $p_S<0.0001$ $p_{T*S}<0.0001$
	OTA	1777 ± 55^{aA}	1585 ± 47^{bB}	1510 ± 47^{bA}	1328 ± 41^{cC}	
	Fhx	1815 ± 46^{aA}	1531 ± 53^{bB}	1454 ± 45^{bAB}	1469 ± 45^{bB}	
	Pyr	1498 ± 49^{cB}	1720 ± 47^{aB}	1411 ± 44^{bcB}	1553 ± 48^{bB}	

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L)

*a, b, c, d; significant difference at $P \leq 0.05$ (mean values with the same letter in the same line do not differ significantly; effect of strain)

*A, B, C, D; significant difference at $P \leq 0.05$ (mean values with the same letter in the same column do not differ significantly; effect of treatment)

*the statistical analysis of samples were analyzed applying the mean SD for each fermentation product from analysis method calibration to analyzed results of samples (see materials and methods)

Removal of Pyrimethanil, Fenhexamid and Ochratoxin A in Fermentative Assays During Alcoholic Fermentation and After Extended Contact With Wine Yeasts

Yeast strains were found to remove contaminants from grape juice as well as synthetic media in fermentative and stationary assays.[11, 15, 18, 33, 39, 88] In previous studies it was demonstrated that Pyr, Fhx and OTA can be removed by cell wall mannoproteins adsorption [14, 15, 24, 33], but not by degradation in synthetic media and in grape juice, in contrast to some other pesticides.[89, 90]

In Table 4 the removal capacity of four genetically different yeasts for Pyr, Fhx and OTA in cv. Sauvignon Blanc grape juice/wine can be seen. The adsorption potential was evaluated after alcoholic fermentation and the extended contact between yeasts and media containing contaminants since, Nunez and colleagues[24] and Bizaj and colleagues[15, 33] demonstrated that the main release of mannoproteins from the yeast cell wall occurs within seven days after the end of fermentation when they also adsorb a fraction of contaminants. However, the work of Bejaoui and colleagues[14] showed that contaminants such as OTA can be released back into synthetic media after being adsorbed onto yeast components. Our results confirmed this in all assays except for AWRI 1640 as the amount of removed OTA was significantly higher after the extended contact. The partially removed contaminants after alcoholic fermentation were of a higher proportion for all yeast strains if compared to previous works in synthetic media.[15,18,33,91] This way suggesting the importance of environmental conditions for the adsorption capacity of yeast cell walls, especially pH, which determines the charge of functional groups on mannoproteins and binding contaminants.[11, 16, 26, 38]
From the results shown in Table 4 it is also evident that the removal potential of yeast strains in fermentative assays is strain and species dependent, which was found also in other studies, however in synthetic media it was not always the case. [15,16,18, 33,37, 39]
In Fhx treatments, AWRI 1640 had the lowest potential for adsorption at the end of the alcoholic fermentation, as well as after the extended contact; the potential of the other three strains was not significantly different after the extended contact.
In Pyr treatments the two *S. cerevisiae* strains (AWRI 1640 and 0838) had the same removal potential after the extended contact time. However this was higher in comparison to the two hybrids where AWRI 1810 in this case showed significantly different, intermediate inheritance from the two parental strains. AWRI 1539 was able to remove only 24.62 % of pyrimethanil. This can be explained by the intensive negative effect of this contaminant on its kinetics and consequently the production of yeast biomass which is crucial for the removal potential.[15, 18, 24, 33,39]

Opposite to Pyr, when OTA was present, the two hybrids had the highest potential for removal; AWRI 1810 shows significantly different, intermediate potential in comparison to parental strains, where AWRI 1640 shows the lowest potential for adsorption.
Different genetic background of the four strains and the induced mutations in AWRI 1640 define yeast cells morphologically, chemically and metabolically.[26, 27, 48, 92] The sensitivity of strains to contaminants[15, 33, 35, 38, 91], and the composition of yeast cell walls[26, 27, 92] define importantly the removal potential. However the shape and the volume/surface rate of yeast cells[16, 93] as well as the dynamics of autolysis[14, 24, 31, 32,94] are strain dependent, which means that these can contribute to the strain dependent removal potential as well. On the other hand, being part of environment where yeast cells were growing, fungicides have varied affinity of binding to different yeast strain. This can be clearly seen in the Table 4; however the physico – chemical conditions of the media affect binding affinity in fermentative and especially extended contact phase; where the contaminants are as well as different genetic background for yeast, of different chemical composition.

Table 4. Removal potential of strains AWRI 1640, 0838, 1539, 1810 for contaminants fenhexamid, pyrimethanil (mg/L) and ochratoxin A (µg/L) in cv. »Sauvignon Blanc« 2005 grape juice assays carried out at 17 °C. The potential was evaluated after the fermentative stage (EF) and after prolonged contact (7 days) between yeast lees and media (PCL). Data reported are mean values and standard deviations of three independent experiments carried out under identical conditions.

Strain	Contaminant	Stage of fermentation		Percentual removal	
		EF	PCL	% removal EF	% removal PCL
1640	Fhx	7.67^{aAn}	7.42^{bAn}	23.32	25.76
0838	Fhx	7.45 ± 0.12^{aA}	6.25^{bBn}	25.55	37.52
1539	Fhx	7.27 ± 0.36^{aAB}	6.40 ± 0.12^{Bb}	27.35	35.99
1810	Fhx	7.00 ± 0.05^{aB}	6.43^{bBn}	30.04	35.66
1640	Pyr	6.85 ± 0.48^{aA}	5.48 ± 0.46^{bA}	31.50	45.22
0838	Pyr	8.62 ± 0.52^{aB}	5.54 ± 0.24^{bA}	13.80	46.30
1539	Pyr	9.00 ± 0.11^{aB}	7.54 ± 0.57^{bB}	10.01	24.62
1810	Pyr	7.82 ± 0.05^{aC}	6.13 ± 0.06^{bC}	21.76	38.68
1640	OTA	3.53^{aAn}	3.38 ± 0.34^{aA}	29.40	32.40
0838	OTA	3.05 ± 0.09^{aB}	1.85 ± 0.02^{bB}	39.00	63.00
1539	OTA	2.46 ± 0.16^{aC}	1.46 ± 0.16^{bC}	50.80	70.80
1810	OTA	2.32 ± 0.14^{aC}	1.64 ± 0.10^{bC}	53.60	67.20

*C=control; OTA=Ochratoxin A (5 µg/L); Fhx= Fenhexamid (10 mg/L); Pyr=Pyrimethanil (10 mg/L) initial concentrations
*a, b, c, d; significant difference at $P \leq 0.05$ (mean values with the same letter in the same line do not differ significantly;
*A, B, C, D; significant difference at $P \leq 0.05$ (mean values with the same letter in the same column (each treatment separatelly) do not differ significantly; effect of strain)
*n=number of replicates is 2

In this study we highlighted the complexity of interactions of genetically different industrial wine yeasts and contaminants, coming from natural spoilage mycobiota on grape berries (OTA) on one, and on the other hand fungicides (pyrimethanil and fenhexamid) working antagonistically against spoilage mycobiota. For the first time OTA and Fhx were demonstrated, and Pyr was confirmed, to negatively affect fermentation kinetics of industrial yeast cells in natural grape juice. However their intensiveness was dependent upon the genetic background of the yeast strain. Contaminant's effect on metabolic pathways dictating aromatic and basic composition of wines was evident in all treatments. Moreover, a metabolic pathway was found to be affected differently by the same contaminant, in different strains. This suggests that these interactions can define the composition of the final product. In our study, OTA was demonstrated for the first time to affect the aromatic and basic composition of wines if present in alcoholic fermentations in grape juice.

The composition of final wines was affected by the yeast removal potential for contaminants. The dynamics of removal was different in comparison to synthetic media for all yeast strains. A significant part was removed already during alcoholic fermentation, and not only after the extended contact of yeast lees and wine. This indicates that physico-chemical composition of

media crucially defines the interaction. The removal was found to be strain dependent as well, which shows that the interaction between yeast strains with contaminants in grape juices dictates the composition of out coming wines.
None of the three contaminants was found to increase the concentration of any compounds known to confer desirable sensory characteristics, on the other hand these were found to increase the concentration of non-desirable compounds. This means that both, the presence of OTA as well as the presence of fungicides have the potential to interfere with fermentation efficiency and wine quality.

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3 GENERAL DISCUSSION AND CONCLUSIONS

The aim of our research was to elucidate how the overall quality of wine; that is its safety and its sensory characteristics, is being affected by interactions that occur during alcoholic fermentation in-between genetically different industrial wine yeasts and contaminants commonly present in grape juices.
Following the hypothesis (A, B, C, D, E) we highlighted new findings that will help to understand and improve the process of alcoholic fermentation in winemaking.
The complexity and multi-factoriality of the interactions made us perform the research in 4 parts. Every part is an independent unit, and at the same time, an unit of the whole research.
The first part; "Removal of ochratoxin A in *Saccharomyces cerevisiae* liquid cultures" describes the interactions in-between the mycotoxin produced by grape infecting fungi, Ochratoxin A (OTA) with wine yeast strains of *Saccharomyces cerevisiae* in synthetic media, in stationary and fermentative assays. Since the concentration of OTA in grape juice is inversely proportional to the concentration of botriticides, the second part; "Removal of pyrimethanil and fenhexamid in *Saccharomyces cerevisiae* liquid cultures" describes the interaction of two fungicides from chemically diverse families with commercially available and wild isolates of wine yeast, in synthetic media in stationary and fermentative assays.
With the first two parts we elucidated the basic interactions in-between yeast strains and the contaminants in order to proceed our research in enological conditions.
Wine yeasts after inoculation dramatically change the composition of grape juice, and during alcoholic fermentation transform it into wine. To diversify and improve the outcomes, various industrial yeast strains are being developed; that is why the third part "A Breeding strategy to harness flavor diversity of *Saccharomyces* interspecific hybrids and minimize hydrogen sulfide production" was performed; where for the first time a new strategy for production of industrial food grade wine yeast strains was highlighted and new *Saccharomyces sensu stricto* triple hybrids (alloploids) were produced with improved enological traits.
In the fourth and final part, »Interactions between industrial yeasts and contaminants in grape juice affect wine composition profile", all the basic data coming from the first two parts and the new hybrids coming from the third part were used in wine production to uncover the types of interactions of genetically diverse starter cultures with contaminants in enological conditions, this way enabling a better understanding and improvement in wine production technology.

3.1 GENERAL DISCUSSION

The quality of food is determined by its safety and by its sensory properties. During alcoholic fermentation, wine yeasts define its quality in the most crucial way; that is why we did study the interactions in-between genetically different strains and contaminants in this phase.

3.1.1 The definition of interactions

In the first two parts we did focus on the analysis of the type of interactions that may occur in-between genetically different strains and three types of complementary contaminants in stationary and fermentative assays, in synthetic defined media; phosphate buffer and yeast extract - malt extract broth (YM) respectively, because we wanted to exclude any type of external undefined disturbing factor to define whether there was an interaction present and its type.

The complementary contaminants were chosen; on one hand pyrimethanil (Pyr), from the anilinopyrimidine class of fungicides (Tomlin, 1994) and fenhexamid (Fhx) from the hydroxyanilides class of fungicides (Tomlin, 1994) used for treatments against mold pests on grapes, and on the other ochratoxin A, a mycotoxin whose chemical structure consists of a chlorine-containing dihydroisocoumarin linked through the 7-carboxyl group to l-α-phenylalanine (Caridi, 2007) produced by two genera of fungi on grapes: *Aspergillus* and *Penicillium* (Mateo et al., 2007).

Because of the health-threatening properties of the three chemical compounds to humans, their concentration in media should be as low as possible.

In the first part of the study, we analyzed the capacity of the removal of OTA by a commercially available wine yeast *Saccharomyces cerevisiae* Lalvin EC-1118 and a wild yeast isolated from a spontaneous fermentation of cv. Malvasia grape juice, *Saccharomyces cerevisiae* ZIM1927. The type of interaction was analyzed as well, because there was no firm consensus about the proposed mechanism involved in the removal of OTA (Bejaoui et al., 2004; Caridi, 2006; Cecchini et al., 2006; Caridi, 2007; Hocking et al., 2007). In the same trend, we did analyze the capacity and the mechanism for removal of the two pesticides; pyrimethanil and fenhexamid in the second part, to define and clarify the type of interaction.

In the stationary assays we tested; whether OTA, pyrimethanil and fenhexamid can be removed by the yeast biomass and its mechanism; with viable and non-viable (yeast cells inactivated by Na-Azide, which does not affect cell-wall integrity) yeast biomass (described on pages 14 for OTA and 21 for Pyr and Fhx). We could confirm the hypothesis B, that yeast biomass was able to remove OTA from the media, at the same time we could see, that there was no significant difference between the capacity of removal of viable and non-viable biomass, which strongly suggests, that OTA is not degraded by the yeast metabolism and that adsorption of OTA is a likely mechanism to account for its removal. For Pyr and Fhx hypothesis B can be confirmed as well, the tests were carried at three different concentrations of contaminants (0,1; 1,0; 10,0 mg/L) and at all three, the pesticide was removed. There was no difference in removal capacity between viable and non-viable cells, suggesting as in the case of OTA, that the removal is due to adsorption only.

The hypothesis D can be confirmed in the stationary assays; the removal is strain dependent, for OTA and the two pesticides as well, because even though all the strains are *S. cerevisiae*, the composition of the cell-wall is strain dependent (Huwig et al., 2001) and defines the binding potential (Caridi, 2006; Caridi, 2007). However, when very low concentrations of contaminants are present, the strain dependent removal capacity cannot be seen.

The removal is contaminant type dependent as well; this can be confirmed for all three contaminants.

In the fermentative assays (described on pages 14 for OTA and 21 for Pyr and Fhx) we could observe that by the end of fermentation (when there was less than 2g/l of reducing sugars in the media), OTA was not significantly removed from the media, only after 7 days of extended contact with yeast lees; the time of the release of the main proportion of mannose, which acts as the main binding agent for the contaminants (Caridi, 2006; Nunez et al., 2007), OTA was removed from media, which confirms the hypothesis C. This contrasts with the work by Bejaoui et al., (2004), however, the work conditions were different; pH, media, strains, are essentially defining adsorption (Ringot et al., 2005; Caridi, 2006; Caridi et al., 2006; Caridi, 2007), as we have demonstrated that is the mechanism for OTA removal.

For Pyr and Fhx was performed the fermentative assay in the same fashion but at three concentrations of contaminants (0,1; 1,0; 10,0 mg/L). The final concentration in the media was only evaluated after the completion of fermentation and the 7 days of extended contact,

because in tests (data not shown) the removal after the completion of fermentation was not significant, confirming the hypothesis C.
In addition, between the two pesticides, Fhx was removed more efficiently from media by both strains; however the difference can be seen only in the assays with the highest initial concentration of contaminants, at lower concentrations the removal is roughly similar.
The hypothesis D for the fermentative assays can be confirmed only partially; the removal capacity was demonstrated to be dependent upon contaminant type but not the strain type, for all the contaminants. This suggests that the environment plays an extremely important role in the adsorption capacity.
In fermentative assays, during the interaction between yeasts and contaminants the action that yeasts take towards the contaminant is always coupled with the contaminant's reaction towards the yeast. To test the hypothesis E and F we performed the basic analyzes of the contaminants potential effects on the yeast strains in synthetic defined media during fermentative assays.
In the first part (Table 3, page 17) OTA was found to affect the fermentation capacity and the production of volatile acidity. This is the first time that OTA was found to affect the metabolic processes in wine yeasts during alcoholic fermentation in synthetic media. At high concentrations of OTA the production of volatile acidity, which is usually considered a stress response during alcoholic fermentations (Pretorius, 2000; Ribereau-Gayon et al., 2006; Oliva et al., 2008), was found to be strain dependent as well. During the tests, this was not expected, since the presence of OTA in these conditions, did not affect the fermentation kinetics at all.
In the second part (Table 2, page 24) only Pyr was able to affect the production of volatile acidity during alcoholic fermentation, and only at the highest initial concentration. Its toxicity has been mentioned before (Čus and Raspor, 2008), and the sensitiveness toward Pyr was found to be strain dependent as well. The commercially available yeast produced significantly higher amounts of volatile acidity.
On the other hand it was surprising that Fhx did not significantly affect the production of volatile acidity, because by the irregularity of fermentation curves (Figure 2b, page 24) it could be observed that as the initial concentration of Fhx increased, the fermentation kinetics decreased but anyway reaching the fermentation dryness. A similar trend was observed for Pyr as well; however its negative effect on the kinetics was stronger in comparison to Fhx (Figure 2b, page 24).
It can be observed; that ZIM1927 was more sensitive, because when the highest initial concentration of Pyr was present, the fermentation remained stuck.
These results suggests that the toxicity of Pyr, has defined the lower removal of Pyr from media in comparison to Fhx which is less toxic, suggesting that lower amounts of biomass were produced, which is crucial for contaminants removal (Caridi et al., 2006; Caridi, 2007).
With these results in our conditions we have found that OTA and Pyr (and not Fhx) are able to affect the wine yeast metabolome during fermentations and that its expression is strain and contaminant dependent, partly confirming the hypothesis F. On the other hand we can partly confirm the hypothesis E, because Pyr and Fhx (and not OTA) are able to affect fermentation kinetics and its expression is strain and contaminant dependent.

3.1.2 The improvement of industrial wine yeasts

Mycotoxins and pesticides are harmful to human health (Cabras and Angioni, 2000; Battilani et al., 2006), moreover, some pesticide's negative effect on starter cultures in wine production have been found as well (Oliva et al., 1999; Garcia et al., 2004; Oliva et al., 2008; González-Rodríguez et al., 2011; Noguerol-Pato et al., 2011). In the first two parts of this thesis, the negative effect of ochratoxin A on starter cultures has been shown for the first time along with

the negative effect of pyrimethanil and fenhexamid and additionally the presence of pesticides and mycotoxins is almost inevitable in grape juices (Cabras and Angioni, 2000)

The flavor diversity and complexity of wine is defined by its fermenting flora, and the most intensive diversity can be reached by spontaneous fermentation, where numerous interactions in-between natural present flora define the sensory properties and the quality of wine (Pretorius, 2000; Fleet, 2003). However, the naturally present flora is not stabile, and cannot give the reliability in production to have constant quality (Ribereau-Gayon et al., 2006). It is also very sensitive to various pesticides present in grape juice (Čuš and Raspor, 2008), in comparison to more stabile industrial strains.

The purpose of the third part of this study was to improve the industrial strains the way to retain their stability, reduce the production of off-flavors and improve the flavor diversity and complexness by the interaction of *Saccharomyces sensu stricto* strains; to confirm the hypothesis A.

The improvement of wine yeasts by generating interspecies hybrids of *Saccharomyces sensu stricto* group has been recently highlighted (Bellon et al., 2011), and studies show that the out-coming hybrids have good aroma production characteristics. Moreover, the presence of *S. kudriavzevii* genome in hybrids was shown to positively affect aroma compound production during alcoholic fermentation (Swiegers et al., 2009; Bellon et al., 2011).

AWRI 1539 (*S.cerevisiae* x *S.kudriavzevii*), an isolate of the commercial strain VIN7 was found to be comprised of diploid *S.cerevisiae* chromosomes and an almost complete complement of *S. kudriavzevii* chromosomes (Borneman et al., 2012). The alloploid AWRI 1116 (*S.cerevisiae* x *S.kudriavzevii*) does not have it's genomic composition completely determined, however the preliminary data infer an incomplete complement of *S.kudriavzevii* chromosomes (Jenny Bellon, personal communication).

These two strains are showing important potential for the production of desirable volatile compounds (Table 1, page 31), on the other hand their potential for H_2S production is very high. Cordente et al., in (2009) produced the first food grade low-H_2S producing mutants of *S. cerevisiae* Mauri PDM® an industrial strain; AWRI 1640, which is the lowest identified producer of H_2S was found to be a mediocre producer of desirable aroma compounds during alcoholic fermentation (Table 1, page 31). AWRI 1640 has a heterozygous point mutation (G176A) in the *MET10* gene, which encodes the α-subunit of the sulfite reductase enzyme, a part of the *sulfate reduction sequence* (Takahashi et al., 1980; Stratford and Rose, 1985). This mutation confers the inactivation of the enzyme and lowers the production of H_2S, but on the other hand, during alcoholic fermentation the production of SO_2 by the strain is high (Hansen and Kielland-Brandt, 1996; Cordente et al., 2009). This can cause problems to the following malolactic fermentation (Versari et al., 1999).

The three parental strains were chosen because of their positive oenological traits and since only non-GMO strains are considered to be food-grade (Pretorius, 2000; Schilter and Constable, 2002), we had to use a classic technique to produce new, food-grade industrial strains.

Even though interspecies hybrids are considered a dead end and additionally, in our sporulation and spores viability tests (described on page 29), we were not able to isolate a single viable spore out of 18 tetrads of the two parental alloploids, it was previously found that these can potentially undergo further hybridization (de Barros Lopes et al., 2002). AWRI 1640 is a mutant obtained with a non-specific mutagenic method (Cordente et al., 2009), which may have affected the reproduction capacity; also in this case we were not able to isolate a single spore out of 18 tetrads; thus we were reliant upon mass-mating.

Mass mating AWRI 1640 X AWRI 1539 and AWRI 1640 X AWRI 1116 was performed (described on page 29) and the selection of potential new hybrids was based upon

complementary phenotypic markers, which were found for all the three parental strains (described on page 29).

AWRI 1640, due to the effect of the non-specific mutagenic treatment that it was exposed to, was incapable to use galactose as carbon source; on the other hand AWRI 1539 was unable to grow at 37 °C and AWRI 1116 was sensitive to low pH in the media (Table 2, page 32). It is important to note, that through the selection of a phenotype such as thermotolerance for selection, shown to be polygenic in industrial strains of *S. cerevisiae* (Marullo et al., 2009), we may have excluded some hybrids with desirable properties.

The phenotypic selection of potential hybrids for low H_2S production on BiGGY plates preceded the genotypic characterization of new hybrids (described on page 32); where we defined as hybrids, strains with mixed inheritance of parental genomes; AWRI 1808, 1809 and 1810 (AWRI 1640 X AWRI 1539) and AWRI 1811, 1812 (AWRI 1640 X AWRI 1116) (Figure 1, page 33). Our hybrids are expected to display extensive aneuploidy, because the genome stabilization usually involves translocation, segmental duplication and even chromosomal loss (Antunovics et al., 2005). Aneuploidy is a common feature of industrial strains, which gives stability and capability to work in harsh industrial conditions; the presence of toxic contaminants as well (Pretorius, 2000; Borneman et al., 2012). However we confirmed that all hybrids contain at least a part of the genome of each of the parental strains. All five selected hybrids contained both *MET10* alleles from the low-H_2S-producing strain and at least one *S. cerevisiae* allele from the flavor-active parent, in addition to the *S. kudriavzevii MET10* allele (Figure 2, page 33).

The presence of the *MET10* G176A allele, found in all five hybrids strongly indicates that this mutation is responsible for the low H_2S phenotype of all the new hybrids, as was previously found (Cordente et al., 2009), moreover these results suggest that the mutation has a strong dominant effect, not only over the *S. cerevisiae* alleles but over *S. kudriavzevii* alleles as well. This means that AWRI 1640 can be used as a tool for production of new hybrids with low H_2S phenotype combined with other industrial traits.

The novel strains were tested in laboratory scale fermentations in defrosted sterile filtered grape juice of chardonnay, to confirm the phenotypes that yeast strains were showing on defined synthetic media (described on page 30).

The volatile fingerprinting (described on page 30) of wines made with new and parental strains analyzed by PCA for the binned mass spectroscopy data for wines, showed that the parental strains clustered separately and reflect the fermentation products profiles (Figure 5, page 35). On the other hand, the new hybrids exhibited intermediate clustering; in line and consistent with other parameters analyzed during fermentation such as fermentation kinetics, SO_2/H_2S production, and the main fermentation parameters of wine for each wine produced by the single strains (Table 3, page 35).

During fermentations with new strains in grape juice, we confirmed the low H_2S production phenotype observed on the BiGGY plates; moreover, with our hybridization approach we kept SO_2 production low (Table 3, page 35). It can be also observed that H_2S production during fermentation in comparison to the alloploid parental strains was dramatically lowered, this way making the production of SO_2 higher. The most suitable strain for further industrial application from this point of view would be AWRI 1810, for which the production of H_2S was roughly, only 15% of the parental strain AWRI 1539 and its SO_2 production would be acceptable for malolactic bacteria for further malolactic fermentation (MLF). For wines where the MLF is not needed AWRI 1808 and AWRI 1809 would also be suitable.

All the fermentations were completed to dryness by all the new strains moreover, an unexpected positive feature was found in all new strains; an extremely low potential for

production of acetic acid - volatile acidity, which at high concentrations could cause spoilage of wine (Table 3, page 35).
With our work we highlighted a novel strategy of combination of classical breeding methods and successfully used it, to hybridize for the first time industrial flavor-active alloploids (*S. cerevisiae* x *S. kudriavzevii*) with an industrial mutant of *S. cerevisiae* with low H_2S phenotype, harnessing for the first time laboratory produced food-grade triple hybrids.The newly produced hybrids are new industrial food-grade strains expressing good fermentation performance (Figure 3 and 4, page 34) producing high concentrations of flavor active compounds and low concentrations of off-flavors (Table 3, page 35). This way we confirmed the hypothesis A.

3.1.3 Interactions of genetically diverse industrial wine yeast strains with complementary contaminants during grape juice alcoholic fermentation

A lot of effort has been put into vine growing and winemaking to follow directions given by international authorities and consumers' requests for a more health responsible production of wine. The given directions are lowering the usage of pesticides and also trying to keep the level of various pests and their harmful products low (EC Regulation, 2005). To preserve safety of wine and improve its sensory complexity and quality, the factors that affect these features, have to be as clear as possible.
The fourth part of the research studied the interactions between complementary contaminants; pyrimethanil (Pyr) and fenhexamid (Fhx) on one hand and ochratoxin A (OTA), which is more present in grape juices produced with less treatments against microbial spoilage (Lo Curto et al., 2004; Pardo et al., 2005; Ribereau-Gayon et al., 2006) on the other, with genetically different industrial wine yeast strains in grape juice. The main reason was to establish, whether the abusive use of fungicides and their presence affects more negatively the final product, than the presence of pests and their secondary products such as OTA.
The industrial strains chosen were: AWRI 0838 (*S.cerevisiae*) an isolate of Lalvin 1118-EC, AWRI 1539 (*S. cerevisiae* x *S. kudriavzevii*) an isolate of VIN7, AWRI 1640 the low H_2S mutant of *S. cerevisiae* Mauri PDM® (Cordente et al., 2009) and AWRI 1810 ((*S. cerevisiae* x *S. kudriavzevii*) x (*S.cerevisiae*)) the triple hybrid produced in the third part of this research.
We used the strain Lalvin 1118-EC as the reference strain throughout the whole research project.
The fermentations were performed in cv. Sauvignon Blanc grape juice (composition described on page 41, Media and Reagents), and the fermentation assays were of the same design as those in fermentative assays in the first two parts of this research. We used only the high initial concentrations of contaminants, that is Pyr=10,0 mg/L; Fhx=10,0 mg/L and OTA=5,0 µg/L, because from the preliminary parts of this research, these were the only concentrations at which we were able to detect interactions. However, since the media was different, differences in interactions in comparison to those happened in the YM (yeast extract-malt extract broth) between yeasts and contaminants were, highly expected.
From the very beginning during the alcoholic fermentations, it could be observed that the 4 different industrial strains have had significantly different kinetic curves, showing that AWRI 1539 was the fastest, AWRI 1640 the slowest, and AWRI 1810 and 0838 intermediate; however all were able to finish the fermentation to dryness. On the other hand, there was no doubt about the negative effect of the three contaminants, where Fhx was less negatively affective in comparison to OTA and especially Pyr, which caused the fermentation of strain AWRI 1539 to remain stuck (Figure 1; page 44; Figure 2, 3 page 45; Figure 4, page 46). In comparison to what we observed in the first two parts of the study, here we demonstrated that

OTA is able to impair the fermentation kinetics, more negatively, if compared to Fhx; which in YM media was affecting fermentation kinetics more than OTA, whose effect could not be found. In this study, OTA and Fhx were found for the first time to negatively affect genetically different industrial strains in grape juice during fermentation. The phenomena, that strains with faster kinetics are more sensitive to contaminants can be confirmed in grape juice, it was also found before in defined synthetic media, suggesting their lower fermentative stability in harsh industrial conditions. The trend of negative affection of different contaminants for each strain can be observed to be different, this way confirming the hypothesis E. Moreover, from the results that we obtained, the environment is defining interactions in-between strains and contaminants drastically, if we compare fermentative assays in synthetic media and grape juice.

Since we have found that in synthetic defined media, contaminants are able to affect certain metabolic processes; in the fourth part of the study we have widely analyzed their effects on genetically different industrial strains during alcoholic fermentation, to see how these affect wine composition and its quality.
The principal chemical compounds, or the basic technological data after the grape juice fermentation as well as volatile fermentation products produced by yeast strains define wine quality along with its safety. All these factors are affected by yeasts, which is why we studied the effect of contaminants on both compositions (Fleet, 2003; Ribereau-Gayon et al., 2006).
The basic technological data (Table 2, page 48), with the exception of the concentration of tartaric acid are compounds produced from the primary metabolism, and since kinetics was negatively affected, we expected lower concentrations of the analyzed compounds produced in comparison to the control assays. The final concentration of malic acid in the media was particularly interesting; the results that we obtained suggest that AWRI 1640 might have affected the malic degradation enzyme because of the highest concentration of malic acid in media in all treatments (Mayer and Temperli, 1963; Radler, 1993). This might be the consequence of the non-specific mutagenesis by which AWRI 1640 was produced (Cordente et al., 2009). Additionally, the higher concentration of malic acid in the wine produced by AWRI 1810, confirms a mixed inheritance. A similar negative effect and strain dependency on the production of citric acid, lactic acid and succinic acid which is particularly important for sensory quality (Ribereau-Gayon et al., 2006) was found as well.
The concentration of acetic acid, whose presence in high amounts can negatively affect the sensory characteristics of wine, was of particular interest since we found that AWRI 1810 is a particularly low producer of acetic acid. Surprisingly, we could not find any effect of the contaminants on all strains except for AWRI 1640, where the effect of the contaminants was negative toward the production of acetic acid.
The production of glycerol, the compound mostly credited for the “body” of wine, was negatively affected when a contaminant was present for all strains, however AWRI 0838 and 1810 were less sensitive to compounds, moreover AWRI 1810 seemed to be stimulated by Pyr; suggesting that Pyr prolonged the initial phase of glycerol production (Ribereau-Gayon et al., 2006), this way lowering the final concentration of ethanol. Ethanol production was negatively affected in line and consistently with the fermentation kinetics, for all strains. AWRI 1539’s production in the case of Pyr was the lowest, in line with the stuck fermentation kinetics observed previously.

Fermentation products, besides the basic chemical composition, are determined by wine yeast strains and the chemical composition of media (Fleet, 2003; Ribereau-Gayon et al., 2006). The

interactions between genetically different backgrounds of yeast strains and contaminants in the same media were expected to produce different outputs (Table 3, page 51).

The ethyl esters are formed during alcoholic fermentation through enzymatic formation between free alcohols and the acyl-S-CoA (Shinohara and Watanabe, 1981). Ethyl acetate, the main ester was expected to be produced in lower amounts, since the amounts of ethanol produced were lower for all strains when contaminants were added. In effect this was the case; the negative affection trend was in line with fermentation kinetics and ethanol production. Similar results were obtained for ethyl hexanoate; the two esters are very important for proper sensory characteristics in wines, and both were produced over the perception threshold (Robichaud and Noble, 1990; Garcia et al., 2004). On the other hand ethyl propanoate and ethyl butanoate were not, the latest was negatively affected, for all strains. Ethyl propanoate was stimulated for all strains, except for AWRI 1810 whose production was lowered, suggesting that OTA has an especially strong negative effect.

Ethyl 2-methylpropanoate was stimulated by the presence of all the contaminants, even strengthening the negative effects that it has on wine sensory properties. Other two negative indicators were also analyzed; ethyl 2-methylpropanoate and ethyl 3-methylpropanoate (Shinohara, 1985; Edwards et al., 1990), the latest could not be detected, on the other hand, ethyl 2-methylpropanoate was detected at minimum concentrations, and was strain dependent and negatively affected by all contaminants.

All the acetates were detected under their sensory threshold (Garcia et al., 2004). 2-phenyl ethyl acetate confers to young wines the flavor of roses and violets. Its production was strain dependent and negatively affected by all contaminants especially Pyr; the AWRI 0838 strain was the most sensitive to Pyr and has produced a non-detectable concentration of it. The other acetates analyzed; 3-methyl butyl acetate, 2-methyl butyl acetate and 2-methyl propil acetate are very important for young wine flavor; their production was strain dependent and negatively affected by all contaminants, especially Pyr. Similar trend can be observed for hexyl acetate, with the exception of OTA, not being able to affect AWRI 1539.

The important group of volatile compounds that affect sensory characteristics of wine are higher alcohols, which are produced by yeast strains during alcoholic fermentation from amino acids and sugars, which are their precursors (Schreier, 1984; Giudici et al., 1990; Dubois, 1994). The isoamilyc alcohols 2-methyl butanol, 3-methyl butanol, and 2-methyl propanol confer fruity flavor and intensity to wine; their production was found to be strain dependent and the hybrids were the highest producers. All the contaminants negatively affected the production of isoamilyc alcohols, all were found in concentrations higher than their perception threshold (Oliva et al., 1999).

The only higher alcohol that was found under the perception threshold in wine was hexanol, which confers to wines herbaceous flavor. The production was strain dependent; where AWRI 1640 was the highest producer, and was negatively affected only in the case when Pyr was present. On the other hand in all other assays where a contaminant was present, there was a negative effect on the production of hexanol.

Besides positive flavors produced by the yeast's secondary metabolism, in our studies, we focused on the production of the off-flavor, H_2S during fermentation (Table 1, page 47).

The problem of H_2S production was solved in the third part of our study, when we developed the strain AWRI 1810; which is why we gave particular attention on the effect of the contaminants on H_2S production during fermentation. As expected in fermentations where AWRI 1640, was used there was no detectable H_2S produced. We found that in the case of AWRI 0838 and 1539 the amount of H_2S produced was the highest in control fermentations where no contaminant was added and as expected, since the contaminants negatively affected kinetics, these also negatively affected H_2S production (Cabras et al., 1995; Edwards and

Bohlscheida, 2007). Interestingly, for the newly produced triple hybrid AWRI 1810, Pyr and Fhx significantly stimulated the production of H_2S; on the other hand, OTA had no effect on this strain. This suggests that Pyr and Fhx might have an effect on the complex catabolic system of H_2S production in this strain.
We can observe that the contaminants Pyr and Fhx on one hand, and OTA on the other, have a very important effect on the yeast metabolome during fermentation assays in grape juice. The intensity varied depending on the strain and the type of contaminant, confirming the hypothesis F for fermentation assays in grape juice. This means, that the interactions between genetically different backgrounds and contaminants produce different outputs. At the same time we can observe that none of the contaminants showed positive effects on the chemical composition of wine; which means that neither the intensive use of pesticides nor the absence of treatments, meaning higher amounts of infections of pests is a good practice. The industrial strains that we've tested, including the newly produced triple hybrid AWRI 1810 showed fermentation stability and their capacity for resistance to high concentrations of contaminants as well. There is a need for very careful screening of conditions for pests' infection of grapes and to make accurate treatments to prevent infections and reduce as much as possible the residual amounts of pesticides on grapes.
The problem of residual amounts of pesticides and mycotoxins on grapes, and the possibility to remove them from the grape juice was studied in the last part of the study. Although some of these are removed during initial wine processing procedures, their concentration in grape juice can be still a threat (Miller et al., 1985; Tsiropoulos et al., 2005; Kaushik et al., 2008; Čuš et al., 2010).

Since we have demonstrated in the first two parts of this study that wine yeasts are able to remove contaminants in fermentative and stationary assays in synthetic media, and that the mechanism for their removal is adsorption only, we evaluated their capacity in enological conditions (Table 4, page 54).
Due to the change of media, and the same fermentation design, we decided to analyze the removal potential in two phases; after the fermentation reached dryness/stuck, and after 7 days of extended contact with yeast lees ("battonage"), because it was previously demonstrated by Nunez et al., (2007) and confirmed in the first part of this study that the main release of mannoproteins, the main binding agents for contaminants happens during this period, defining the total removal potential. The results we obtained for all contaminants, confirm what was found during fermentation assays in synthetic media, that the type of media defines the contaminants' removal. The environmental conditions, define the removal capacity, especially in the case of physic-chemical mechanisms of removal (Cabras et al., 1999; Caridi, 2006; Caridi et al., 2006; Angioni et al., 2007). We found a significantly different removal capacity for AWRI 0838 in the two different media, as the reference strain. In grape juice we found that a part of the contaminant present can also be removed during fermentation, not only after, as was the case in the synthetic media. The removal of contaminants in two stages again confirms the hypothesis B and C, because the removal was found to be significantly different in the two phases. In comparison to the fermentative assays in synthetic media, we had a higher genetic diversity and with the results that we obtained, we are able to confirm completely the hypothesis D. The genetic background defines the removal capacity by the sensitivity toward contaminants of the strain, the chemical composition of yeast cell walls and the capacity of mannoprotein production, the shape and the volume/surface rate of cells and the contact time and dynamics of autolysis of yeast cells (Cabras et al., 1999; Martínez-Rodriguez et al., 2001; Martínez-Rodríguez et al., 2001; Cabras et al., 2003; Bejaoui et al., 2004; Caridi, 2006;

Mazauric and Salmon, 2006; Caridi, 2007; Nunez et al., 2007; Čus and Raspor, 2008; Gonzalez-Ramos et al., 2008).
The removal of Pyr by AWRI 1539 was a classic example, when the sensitivity toward the contaminant is high, and the impossibility of production of higher amounts of biomass does not enable a higher removal. Moreover, AWRI 1810 can be seen to have the removal capacity intermediate in comparison to the parental strains, however in line with the results in previous parts of this work.
The removal for the other contaminants was dependent on yeast strains, as well as the affinity of contaminants toward specific strains was contributing to the final removal (Table 4, page 54).

3.2 CONCLUSIONS

From the results obtained in all the four parts of the study the main conclusions are the following:

- the strategy used for improvement of industrial yeast strains, enabled the production of triple alloploid hybrids showing low H_2S phenotype and intensive production of desirable flavors , as well as solid fermentation kinetics;

- industrial and wild yeast strains can remove pyrimethanil, fenhexamid and ochratoxin A from synthetic media and grape juices in stationary and fermentative conditions, and the mechanism for their removal in media does not implicate metabolic degradation; adsorption is the only suggested mechanism;

- the removal capacity of metabolically active biomass is significantly different during the fermentation stage than the metabolically active biomass after the end of fermentation, and is dependent upon strain and contaminant type and the type of media (environment) in which the fermentation is carried out;

- high concentrations of ochratoxin A, pyrimethanil and fenhexamid are able to negatively affect the fermentation kinetics of genetically different industrial yeast strains; in order from the most negative: pyrimethanil, ochratoxin A and fenhexamid. Yeast strains had a different response to different types of contaminants, however the type of media (environment) in which the fermentation is conducted was an important factor;

- high concentrations of ochratoxin A, pyrimethanil and fenhexamid are able to affect the exo-metabolome of genetically different industrial yeast strains during alcoholic fermentation; the interactions in-between different genetic backgrounds of yeasts and contaminants gave different outputs, however the environment defined these as well.

Besides the main conclusion we found that:

- AWRI 1640 was found to be a good tool that can be used in strategies for lowering H_2S production during alcoholic fermentation in food grade industrial yeasts;

- the point mutation in the *MET10* gene (G176A) in AWRI 1640 strain is dominant;

- it is possible to retain low SO_2 production during alcoholic fermentation with yeast strains having a non-active sulfite reductase enzyme;

- novel triple hybrids have low production potential for volatile acidity;

- in sterile grape juice the removal of contaminants by yeasts was more effective in comparison to the YM (yeast extract – malt extract broth), moreover in YM there was no significant removal after the end of fermentation, the removal was all performed in the extended contact phase;

- all media were found to have a higher level of safety after the end of alcoholic fermentation because of the interaction between yeasts and contaminants, yeasts removed the toxic contaminants from the media;

- the effect of contaminants toward kinetics and yeast exo-metabolome was found to be more intense during fermentation in grape juice in comparison to YM;

- the negative effect of contaminants on fermentation kinetics is more intense toward yeast strains that have faster fermentation rates;

- the contaminants were found to negatively affect wine sensory properties, that means that all three contaminants have a negative effect on the quality of wine;

- the optimal quality of wine can be achieved with industrial strains with good enological traits and the lowest possible treatments with pesticides during the growing season that still prevent pests infection and the production of their side products, because no matter if the phytopharmaceuticals are complementary to ochratoxin A, all of these negatively affect the quality of wine; its safety and sensory properties.

4 SUMMARY (POVZETEK)

4.1 SUMMARY

Wine is not considered to be a commodity; however with the current global economic situation and its partial degradation, to everyday consumption food, its safety and its smell are the most common features of wine that are requested by an average wine consumer. Since alcoholic fermentation mainly defines the quality of wines, and wine yeasts are conducting it, we studied the interactions of wine yeasts and commonly present complementary contaminants; pyrimetahnil and fenhexamid which are fungicides used in treatments during the grape growinh season and ochratoxin A, a mycotoxin produced by mold pests on grapes. All are harmful to humans, and because of their presence in grape juices before alcoholic fermentation they may affect wine composition as well.

We found that industrial and wild yeast strains are able to remove all three contaminants in synthetic defined media first and in enological conditions later as well, this way showing that during wine fermentation, wine safety can be improved. The removal potential was different for genetically different wine yeast strains. The mechanism for removal was found to be in all cases adsorption only, because no metabolic action could be observed. Especially in enological conditions we had very high genetic diversity of industrial wine strains; AWRI 0838 (*S. cerevisiae*) (the reference strain also in our synthetic media), AWRI 1539 (*S. cerevisiae* x *S. kudriavzevii*), AWRI 1640 the low H_2S mutant of *S. cerevisiae* and AWRI 1810 ((*S. cerevisiae* x *S. kudriavzevii*) x (*S. cerevisiae*)) the triple hybrid produced in the third part of our study. Because only chemical-physical interactions were found between contaminants and strains, we found that besides the genetic background, contaminants are binding to some strains rather than to other strains, so contaminant affinity was found to be important as well. Moreover, the sensitivity of strains to contaminants was found to be an important factor that defines removal capacity; especially pyrimethanil which was particularly negatively affecting AWRI 1539, and caused its stuck fermentation. The lower amount of yeast biomass produced during fermentation defined lower removal capacity by AWRI 1539. The type of media used for fermentation affected removal potential and the effect of contaminants on the yeasts as well. All contaminants were found to negatively affect fermentation; kinetics and the metabolome. In enological conditions the effect of contaminants was significantly different in comparison to synthetic defined media. The basic technological parameters as well as the fermentation products, which define aromatic compounds, were negatively affected, however it depended on the yeast strain and the type of contaminant. This means that even if the contaminants are complementary, pesticides should be used in concentrations as low as possible during the wine growing season, however in high enough concentrations in order to prevent grapes form pests' infection and their metabolites production, because both are threatening human health and the quality of wine.

Industrial yeast strains are able to ferment in harsh conditions, being resistant to high concentrations of toxic contaminants, and their important and wanted trait is to produce high amounts of desirable flavors and low amounts of off-flavors, such as H_2S. Their negative side is that due to various mutations, deletions, segmental duplications, translocations and even chromosomal losses, their possibility to be improved by classical techniques is very low. Since *S.kudriavzevi*'s genetical background was found to have a positive effect in alloploids with *S. cerevisiae*, we used interspecific hybrids as parental strains to be hybridized with a *S.cerevisiae* mutant for production of low amounts of H_2S, however producing non acceptable amounts of SO_2. By using a novel strategy where mass mating is coupled with complementary phenotypic markers, triple interspecific hybrids were produced for the first time, these are able to express

reliable fermentation performances, and produce high concentrations of desirable volatile compounds and low amounts of H_2S along with acceptable amounts of SO_2. The strategy we used can be applied for other enological traits in industrial yeasts, and AWRI 1640 was found to be an important tool for reduction of H_2S production in industrial yeasts, because of the dominance of the mutation thatinduces the low H_2S phenotype expression. One of the new triple hybrids produced, AWRI 1810 was found to express especially positive enological traits in enological conditions and stability under harsh conditions, having good potential to be industrially applied.

4.2 POVZETEK

Kakovost je opredeljena kot seštevek dobrih in slabih lastnosti neke dobrine. Kakovost hrane in tako tudi vina je določena z njeno varnostjo in senzoričnimi lastnostmi. Med alkoholno fermentacijo, vinske kvasovke močno vplivajo na kakovost vina, zato to delo obravnava medsebojni vpliv med gensko različnimi vinskimi starterskimi kulturami in kontaminanti v tej fazi.

Namen raziskave je bil razkriti kako lahko medsebojni vplivi med gensko različnimi industrijskimi sevi kvasovk in v grozdnem soku prisotnimi kontaminanti, vplivajo na kakovost vina, varnost in senzorične značilnosti.

Na podlagi delovnih hipotez: A) novo razviti medvrstni križanci *Sacharomyces sensu stricto* izražajo pozitivne podedovane lastnosti, B) kvasna biomasa ima sposobnost zmanjšanja koncentracije kontamnantov iz medija, C) sposobnost zmanjšanja koncentracije kontaminantov med alkoholno fermentacijo je različna od sposobnosti zmanjšanja koncentracije kontaminantov po zaključku alkoholne fermentacije s strani žive kvasne biomase, D) sposobnost zmanjšanja koncentracije kontaminantov s strani kvasne biomase je odvisna od seva kvasovk kot tudi vrste kontaminanta, E) visoke koncentracije ohratoksina A in fitofarmacevtsih sredstev v mediju vplivajo na fermentacijsko kinetiko, vplivi so odvisni od seva in vrste kontaminanta in F) visoke koncentracije ohratoksina A in fitofarmacevtsih sredstev v mediju vplivajo na kvasni metabolom, vplivi so odvisni od seva in vrste kontaminanta, smo razkrili nova dognanja, ki bodo olajšala razumevanje in izboljšala proces alkoholne fermentacije med proizvodnjo vina.

Zaradi kompleksnosti teme, smo izvedli raziskavo v štirih delih, od katerih je vsak del individualna enota in obenem del celote. Prvi del; »Odstranjevanje ohratoksina A v tekočih medijih s *Saccharomyces cerevisiae*« opisuje medsebojne vplive med mikotoksinom (ohratoksin A), ki je proizveden na grozdnih jagodah med rastno sezono ob okužbi s plesnimi in vinskimi sevi *Saccharomyces cerevisiae* v sintetičnih medijih v fermentativnih in stacionarnih pogojih. Ker je koncentracija ohratoksina A v grozdnem soku obratno sorazmerna s koncentracijo prisotnih fungicidov, so v drugem delu; »Odstranjevanje pirimetanila in fenheksamida v tekočih medijih s *Saccharomyces cerevisiae*« opisani medsebojni vplivi v fermentativnih in stacionarnih pogojih, med dvema fungicidoma in vinskimi sevi kvasovk, tako komercialnih sevov kot tudi sevov izoliranih iz spontanih alkoholnih fermentacij grozdnega soka.

V prvih dveh delih so bili raziskani medsebojni vplivi med vinskimi sevi kvasovk in kontaminanti v laboratorijskih pogojih, katerih rezultati so postali osnova za nadaljnje raziskovanje v enoloških pogojih.

Vinske kvasovke, po inokulaciji odločilno spremenijo sestavo grozdnega soka, v procesu alkoholne fermentacije ga pretvorijo v vino. Za izboljšavo kakovosti novo nastalih vin, potekajo konstantne raziskave in izboljšave novih vinskih industrijskih sevov; zato je bil v tretjem delu raziskav »Plemenilna strategija za dosego aromatske kompleksnosti in zmanjšanje produkcije H_2S pri medvrstnih hibridih *Saccharomyces* spp« cilj, razvoj izboljšanih industrijskih sevov za dosego višje kakovosti vina. Razvita je bila nova strategija za proizvodnjo izboljšanih industrijski sevov kvasovk in obenem kot popolna novost so bili predstavljeni *Saccahromyces sensu stricto* trojni hibridi (aloploidi) z izboljšanimi enološkimi lastnostmi.

V četrtem in zadnjem delu; »Interakcije med industrijskimi sevi kvasovk in kontaminanti v grozdnem soku vplivajo na sestavo vina«, so bile vse osnovne informacije iz prvih dveh delov in novi hibridi iz tretjega dela uporabljeni v enoloških pogojih za kompleksno razkritje

medsebojnih vplivov med gensko različnimi vinskimi starterskimi kulturami in kontaminanti. Na ta način smo omogočili boljše razumevanje in obenem izboljšavo procesa vinske pridelave.

Medsebojni vplivi kontaminantov in starterskih kultur v sintetičnih medijih

V prvih dveh delih raziskave smo se osredotočili na interakcije med gensko različnimi vrstami vinskih kvasovk ter dvema tipologijama med seboj komplementarnih kontaminantov, fitofarmacevtskima sredstvoma (pirimetanil in fenheksamid) in mikotoksini (ohratoksin A) v sintetičnih medijih; YM (tekoča zmes kvasnega in sladnega ekstrakta z 18 % m/v glukoze) ter fosfatnem pufru. Na ta način smo izločili zunanje moteče faktorje in določili prisotnost in vrsto interakcije. Analize smo izvedli tako v stacionarnih kot tudi fermentativnih pogojih.
Fitofarmacevtski sredstvi, ki sta bili izbrani sta bili pirimetanil (Pyr), ki spada v anilinopirimidinski razred fungicidov (Tomlin, 1994) in fenheksamid (Fhx) iz hidroxianilidnega razreda (Tomlin, 1994); obe sta uporabljeni v programih zatiranja botritisa na vinski trti. Izbrani komplement le-teh pa je ohratoksin A (OTA), mikotoksin, ki je kemijsko di-hidroksikumarin, ki vsebuje klor, vezan preko 7-karbonilne skupine na l-α- fenilalanin (Caridi, 2007). Ohratoksin A na vinskih trtah proizvajata dve vrsti plesni; *Aspergillus* in *Penicillium* (Mateo in sod., 2007).
Zaradi škodljivosti teh kontaminantov za človeka je vinogradniška kot tudi vinarska praksa, da se skuša zmanjšati koncentracijo le-teh v končnem proizvodu.
V prvem delu raziskave smo določali sposobnost komercialnega vinskega seva *Saccharomyces cerevisiae* Lalvin EC-1118 in seva iz spontane fermentacije grozdnega soka sorte Malvazija *Saccharomyces cerevisiae* ZIM1927 za zmanjšanje koncentracije OTA v sintetičnem mediju. Preverjali smo tudi vrsto interakcije med kvasovkami in kontaminantom, kajti ni še povsem jasno, kakšni so mehanizmi odstranitve OTA iz medijev (Bejaoui in sod., 2004; Caridi, 2006; Cecchini in sod., 2006; Caridi, 2007; Hocking in sod., 2007). Na enak način smo preverili interakcije v drugem delu raziskave s pirimetanilom in fenheksamidom, definirali smo mehanizem in potencial za odstranjevanje fitofarmacevtskih sredstev.
V stacionarnih testih v fosfatnem pufru smo preverili, ali kvasna biomasa vpliva na zmanjšanje koncentracije OTA, pirimetanila in fenheksamida v mediju in mehanizem le-tega. Poskusi so bili izvedeni z metabolno aktivno in metabolno neaktivno (inaktivacija z Na-Azid-om; za ohranjanje integritete celične stene kvasovk) kvasno biomaso (opisano na straneh 14 (OTA) in 21 (Pyr in Fhx). Z opravljenimi testi smo potrdili hipotezo B, torej kvasna biomasa vpliva na zmanjšanje OTA v mediju, obenem pa ni bilo opaziti statistično značilnih razlik glede potenciala za zmanjšanje koncentracije OTA med metabolno aktivno in neaktivno biomaso, kar kaže, da OTA ni metabolno razgrajen in da je adsorpcija najbolj verjetni mehanizem odstranjevanja. V drugem delu je bila potrjena hipoteza B tudi za pirimetanil in fenhekasmid; potencial za zmanjšanje koncentracije pesticidov smo preverjali na treh nivojih koncentracij (0,1; 1,0; 10,0 mg/L), in na vseh treh je kvasna biomasa zmanjšala koncentracijo v mediju. Tako kot pri poskusih za OTA ni bilo opaziti statistično značilnih razlik potenciala zmanjšanja koncentracije kontaminanta med metabolno aktivno in neaktivno biomaso, kar še enkrat kaže na to, da v proces zmanjšanja koncentracije kontaminanta v mediju ni vpleten katabolni metabolizem, ampak le adsorpcija.
Delovna hipoteza D je bila potrjena v stacionarnih poskusih. Potencial za zmanjšanje koncentracije kontaminantov je sevno odvisen tako v poskusih z OTA kot tudi z obema pesticidoma. Kljub temu, da sta oba seva vrste *S. cerevisiae*, je sestava celične stene sevno odvisna (Huwig in sod., 2001) in le-ta definira potencial za vezavo (Caridi, 2006; Caridi, 2007).

Na potencial za zmanjševanje koncentracije kontaminantov vpliva tudi vrsta samega kontaminanta, kar se kaže za vse tri. Pri poskusih, izvedenih v fermentativnih pogojih v gojišču YM z 18 % m/v glukoze (opisano na straneh 14 za OTA in 21 za Pyr in Fhx), je bilo opaziti, da se do zaključka alkoholne fermentacije (ko je koncentracija reducirajočih sladkorjev v mediju manjša od 2 g/L) koncentracija OTA v mediju ni statistično značilno zmanjšala, šele po 7 dnevih postfermentativnega podaljšanega kontakta z usedlino kvasne biomase, času, ki je potreben za sprostitev večine manoze iz celičnih sten kvasovk, ki deluje kot glavni agent vezave (Caridi, 2006; Nunez in sod., 2007), je bila koncentracija OTA statistično značilno zmanjšana. S tem je bila potrjena hipoteza C. Rezultati so sicer v nasprotju z ugotovitvami raziskave Bejaoui in sod. (2004), vendar so bili delovni pogoji v naših poskusih različni; pH, medij, sevi kritično definirajo adsorpcijo (Ringot in sod., 2005; Caridi, 2006; Caridi in sod., 2006; Caridi, 2007), ki je dokazano mehanizem za odstranjevanje kontaminantov.

Za pirimetanil in fenheksamid je bil poskus v fermentativnih pogojih izveden v enakih pogojih, vendar so bili kontaminanti ločeno dodani v medij v treh koncentracijah (0,1; 1,0; 10,0 mg/L). Koncentracija kontaminantov je prikazana le po končani alkoholni fermentaciji in sedmih dnevih podaljšanega postfermentativnega kontakta s kvasno biomaso, ker v testih (rezultati niso prikazani) ni bilo mogoče opaziti statistično značilnega zmanjšanja koncentracije kontaminanov po končani fermentaciji. Tako je bila potrjena hipoteza C.

Med dvema pesticidoma je bila koncentracija fenheksamida zmanjšana v večji meri za oba seva, vendar je ta razlika opazna le, ko je bila začetna koncentracija kontaminantov največja, pri manjših koncentracijah začetno dodanih kontaminantov pa je zmanjšanje podobno.

V fermentativnih pogojih je bila hipoteza D potrjena le parcialno; potencialu za zmanjšanje koncentracije je bila namreč dokazana kontaminantna odvisnost, ne pa tudi sevna odvisnost. Ta fenomen kaže na izjemno pomemben vpliv medija na adsorpcijsko aktivnost sevov.

V fermentativnih pogojih so interakcije med kavsovkami in kontaminanti vsi vplivi, s katerimi kvasovke delujejo na kontaminante, in obenem vsi vplivi, s katerimi kontaminanti delujejo na kvasovke. Slednji so bili v preverjanju za potrditev ali zavrnitev delovnih hipotez E in F.

V prvem delu (Preglednica 3, stran 17), se pokaže, da OTA ima vpliv na metabolne procese v kvasovkah med alkoholno fermentacijo v sintetičnih medijih. Ob visoki začetni koncentraciji OTA v mediju analizirani sevi kažejo povečano produkcijo hlapnih kislin in obenem sevno odvisnost za to lastnost; visoka produkcija le-teh med alkoholno fermentacijo je običajno odgovor na negativne motnje iz okolja (Pretorius, 2000; Ribereau-Gayon in sod., 2006; Oliva in sod., 2008). Povečana proizvodnja hlapnih kislin zaradi prisotnosti OTA ni bila pričakovana, kajti le-ta ni značilno vplival na fermentacijsko kinetiko sevov (Slika 1, stran 17).

V drugem delu (Preglednica 2, stran 24) je bilo samo v fermentacijskih pogojih z dodano največjo začetno koncentracijo Pyr značilno, da je pomembno vplival na povečanje proizvodnje hlapnih kislin. Toksičnost pirimetanila je bila potrjena že prej (Čus in Raspor, 2008); v poskusih je bila naknadno ugotovljena sevna odvisnost za občutljivost na ta kontaminant.

V nasprotju s pirimetanilom fenheksamid presenetljivo ne kaže nikakršnega vpliva na produkcijo hlapnih kislin med fermentacijo, predvsem glede na fenheksamidov negativni vpliv na fermentacijsko kinetiko (Slika 2b, stran 24). Navkljub negativnemu vplivu na kinetiko so bile fermentacije ob vseh treh začetnih koncentracijah kontaminantov dokončane. Podoben trend je bil opazen tudi v poskusih s pirimetanilom, vendar je lahko opaziti bolj negativen vpliv pirimetanila v primerjavi s fenheksamidom (Slika 2b, stran 24).

Opaziti je lahko, da je sev ZIM1927 bolj občutljiv na Pyr, kajti ob najvišji začetni dodani koncentraciji pirimetanila je fermentacija zastala.

Rezultati kažejo na to, da je večja stopnja toksičnosti pirimetanila vplivala na manjšo stopnjo odstranitve le-tega iz medija v primerjavi s fenheksamidom, ki se je izkazal kot manj toksičen;

to kaže, da je zaradi večje toksičnosti pirimetanila prišlo do proizvodnje manjše koncentracije biomase v mediju, ta pa je ključnega pomena za zmanjšanje koncentracije kontaminantov.
S pridobljenimi rezultati v danih pogojih je bilo ugotovljeno, da sta OTA in Pyr (ne Fhx) sposobna vplivati na kvasni metabolom med alkoholno fermentacijo, ter da je ekspresija metaboloma odvisna tako od tipa kontaminanta kot od tipa seva, kar delno potrjuje hipotezo F. Tudi delovno hipotezo D lahko le parcialno potrdimo, kajti Pyr in Fhx (ne OTA) imata vpliv na fermentacijsko kinetiko sevov, ekspresija kinetike sevov pa je odvisna tako od tipa kontaminanta kot tudi od tipa seva.

Razvoj novih industrijskih sevov

Mikotoksini so tako kot pesticidi škodljivi za zdravje človeka (Cabras in Angioni, 2000; Battilani in sod., 2006). Za nekatere je bilo dokazano, da negativno vplivajo tudi na starterske kulture v procesu proizvodnje vina (Oliva in sod., 1999; Garcia in sod., 2004; Oliva in sod., 2008; González-Rodríguez in sod., 2011; Noguerol-Pato in sod., 2011). V prvih dveh delih disertacije je bil prvič predstavljen negativni efekt, ki ga imajo ohratoksin A, pirimetanil in fenheksamid na starterske kulture. Prisotnost kontaminantov, tj. pesticidov in mikotoksinov, je v grozdnem soku neizogibna (Cabras in Angioni, 2000).
Na kompleksnost in raznolikost senzoričnih lastnosti vina pomembno vpliva fermentacijska flora v moštu. Največjo stopnjo raznolikosti lahko dosežemo z aplikacijo spontane alkoholne fermentacije, kjer številne in raznolike interakcije med spontano prisotno floro vplivajo in definirajo senzorične lastnosti in kakovost vina (Pretorius, 2000; Fleet, 2003). Negativna stran spontano prisotne fermentacijske flore je nezanesljivost v proizvodnji (Ribereau-Gayon in sod., 2006), pa tudi njena občutljivost na pesticide (Čuš in Raspor, 2008) v primerjavi s stabilnimi komercialnimi industrijskimi sevi.
Namen tretjega dela te naloge je bil izboljšava industrijskih vinskih kvasnih sevov; ohranitev stabilnosti, zmanjšanje produkcije negativnih metabolitov in izboljšanje raznolikosti in kompleksnosti aromatike z interakcijo *Saccharomyces sensu stricto* sevov, s čimer bi se potrdila hipoteza A.
Izboljšanje vinskih kvasnih sevov z generacijo medvrstnih hibridov iz kvasovk *Saccharomyces sensu stricto* je bilo predstavljeno pred kratkim (Bellon in sod., 2011). Iz rezultatov je razvidno, da imajo vsi novo nastali hibridi pozitivne aromatske lastnosti. Izkazalo se je tudi, da prisotnost genoma *S. kudriavzevii* v hibridih pozitivno vpliva na proizvodnjo zaželenih aromatskih spojin med alkoholno fermentacijo (Swiegers in sod., 2009; Bellon in sod., 2011).
AWRI 1539 (*S.cerevisiae* x *S.kudriavzevii*), ki je izolat komercialnega seva VIN7, vsebuje diploidni genom *S.cerevisiae* ter skoraj celotni komplement kromosomov *S.kudriavzevii* (Borneman in sod., 2012). Aloploid AWRI 1116 (*S.cerevisiae* x *S.kudriavzevii*) nima še dokončno determinirane sestave genoma, vendar preliminarni rezultati kažejo, da vsebuje nekompleten komplement kromosomov *S.kudriavzevii* (Jenny Bellon, osebna komunikacija).
Ta dva seva kažeta velik potencial za proizvodnjo želenih hlapnih spojin med alkoholno fermentacijo (Preglednica 1, stran 31), vendar obenem za proizvodnjo H_2S v visokih koncentracijah. Cordente in sod. so v letu 2009 prvič razvili mutante industrijskega seva *S. cerevisiae* Mauri PDM® s fenotipom nizke produkcije H_2S. Izolat iz serije mutantov AWRI 1640, ki je bil identificiran kot najmanjši proizvajalec H_2S, je obenem slab proizvajalec želenih aromatskih spojin med alkoholno fermentacijo (Preglednica 1, stran 31). AWRI 1640 vsebuje heterozigotno točkovno mutacijo (G176A) v *MET10* genu, ki kodira α-podenoto encima sulfit reduktaza, ki je ključnega pomena v »*sulfatni redukcijski sekvenci*« (Takahashi in sod., 1980; Stratford in Rose, 1985). Mutacija vpliva na inaktivacijo encima in zmanjša proizvodnjo H_2S, obenem pa poveča koncentracijo proizvedenega SO_2 med alkoholno fermentacijo (Hansen in

Kiell and-Brandt, 1996; Cordente in sod., 2009). Visoke koncentracije SO_2 so v mladem vinu nezaželene, kajti negativno vplivajo na kasnejšo jabolčno-mlečnokislinsko fermentacijo (MLF) (Versari in sod., 1999).

Trije sevi: AWRI 1539, 1116 in 1640 so bili izbrani za starševske, izrecno zaradi pozitivnih enoloških lastnosti, ki jih imajo. Ker v vinarski proizvodnji lahko uporabljamo le starterske kulture, ki niso gensko spremenjene (ne-GSO) (Pretorius, 2000; Schilter in Constable, 2002), smo za razvoj novih izboljšanih starterskih kultur uporabili kombinacijo klasičnih tehnik.

Čeprav so medvrstni hibridi smatrani kot reprodukcijska »slepa ulica«, poleg tega pa v opravljenih tetradnih analizah pri dveh starševskih aloploidih (opisano na strani 29) ni bila izolirana nobena živa spora iz osemnajstih preverjenih tetrad, je bilo prej dognano, da so aloploidi lahko uspešno nadaljnje križani (de Barros Lopes in sod., 2002). AWRI 1640 je mutant, ki je bil razvit z nespecifično mutagenezo (Cordente in sod., 2009), zaradi katere je lahko prišlo do zmanjšanja reprodukcijskega potenciala; tudi v primeru AWRI 1640 ni bila izolirana nobena živa spora iz osemnajstih preverjenih tetrad. Zaradi dane situacije smo se odločili, da bomo za naš namen uporabili tehniko »mass-mating«.

Po opravljenem »mass-matingu« AWRI 1640 X AWRI 1539 in AWRI 1640 X AWRI 1116 (opisano na strani 29) je bila izvedena selekcija potencialnih novih hibridov na podlagi komplementarnih fenotipskih markerjev, ki so bili identificirani za vse tri starševske seve (opisano na strani 29).

Izkazalo se je, da AWRI 1640 ni sposoben uporabljati galaktoze kot vira ogljika, kar je najverjetneje posledica načina razvoja seva z nespecifično mutagenezo. Obenem sta se nesposobnost seva AWRI 1539 za rast pri 37 °C in občutljivost seva AWRI 1116 na nizek pH medija (Preglednica 2, stran 32) izkazala kot optimalna komplementarna fenotipska selekcijska faktorja. Naj opozorim, da smo s selekcijo na fenotipski lastnosti, kot je termotoleranca, ki je poligenska v industrijskih sevih *S. cerevisiae* (Marullo in sod., 2009), mogoče prezrli nekatere hibride z dobrimi enološkimi lastnostmi.

Sledila je selekcija potencialnih hibridov na potencial za proizvodnjo H_2S na BiGGY gojišču in nato genotipska karakterizacija in definicija hibridov (opisano na strani 32), kjer so bili definirani hibridi kot sevi z vsebnostjo delov genoma obeh starševskih sevov; AWRI 1808, 1809 in 1810 (AWRI 1640 X AWRI 1539) ter AWRI 1811, 1812 (AWRI 1640 X AWRI 1116) (Slika 1, stran 33).

Od novih hibridov lahko pričakujemo ekstenzivno anevploidnost, kajti v procesu stabilizacije genoma so vključene translokacije, segmentne duplikacije ter izgube kromosomov (Antunovics in sod., 2005). Anevploidnost je razširjena lastnost industrijskih sevov, ki omogoča sevom stabilnost in sposobnost za delo v težkih industrijskih pogojih, med drugim tudi ob prisotnosti toksičnih kontaminantov (Pretorius, 2000; Borneman in sod., 2012). Ne glede na potencialno anevploidnost sevov smo v vseh selekcioniranih hibridih potrdili prisotnost vsaj dela genoma iz vsakega starševskega seva. Vseh pet selekcioniranih hibridov vsebuje oba *MET10* alela iz mutanta z nizko proizvodnjo H_2S, pa tudi vsaj en *S. cerevisiae* alel ter *S. kudriavzevii MET10* alel iz aloploidnega starša (Slika 2, stran 33).

Prisotnost *MET10* alela z mutacijo G176A iz starša s fenotipom nizke proizvodnje H_2S v vseh petih hibridih močno kaže na to, da je le-ta odgovoren za fenotip za proizvodnjo nizkih koncentracij H_2S v novih hibridih, kar potrjuje predhodne ugotovitve (Cordente in sod., 2009). Rezultati dodatno kažejo, da je mutacija močno dominantna, ne samo nad aleli *S. cerevisiae*, ampak tudi nad aleli *S. kudriavzevii*. Iz tega izhaja, da se lahko uporablja AWRI 1640 kot orodje za razvoj novih sevov s fenotipom proizvodnje nizke koncentracije H_2S v kombinaciji z drugimi pozitivnimi enološkimi lastnostmi.

Novi hibridi so bili testirani v laboratorijskih fermentacijah v odmrznjenem sterilno filtriranem grozdnem soku sorte Chardonnay za potrditev fenotipov, ki so jih sevi izražali na BiGGY gojišču (opisano na strani 30).
Tako imenovani »volatile fingerpring« ali prstni odtisi hlapnih komponent (opisano na strani 30) vin, proizvedenih iz laboratorijskih fermentacij, ki so bili analizirani s PCA (principal component analysis – analiza glavnih komponent) masnih spektroskopij vin, kažejo, da posamezni sevi grupirajo ločeno in izražajo fermentacijske produktne profile (Slika 5, stran 35).
V fermentacijskih laboratorijskih poskusih v grozdnem soku smo za nove hibride potrdili fenotipe nizke proizvodnje H_2S, ki smo jih opazili na BiGGY gojiščih, še več, z inovativnim hibridizacijskim pristopom smo v nekaterih primerih ohranili tudi nizko proizvodnjo SO_2 (Preglednica 3, stran 35). Opaziti je tudi, da je produkcija H_2S novih hibridov med alkoholno fermentacijo v primerjavi z aloploidnimi starševskimi sevi statistično značilno nižja. Sev, ki se med petorico posebno odlikuje po enološko pozitivnih lastnostih, je AWRI 1810, ki proizvaja le okoli 15% H_2S v primerjavi s starševskim AWRI 1539, obenem pa je proizvodnja SO_2 še vedno tolikšna, da lahko mlečnokislinske bakterije opravijo jabolčno-mlečnokislinsko fermentacijo (MLF). Za proizvodnjo vin, kjer MLF ni predvidena, bi bila lahko primerna tudi AWRI 1808 in AWRI 1809.
Vseh pet novih hibridov je dokončalo laboratorijske fermentacije, poleg tega pa se je pokazala še ena nepričakovana pozitivna lastnost le-teh, to je ekstremno nizek potencial za proizvodnjo ocetne kisline - hlapnih kislin, ki v visokih koncentracijah negativno vplivajo na senzorične lastnosti vina.
V tretjem delu je bila predstavljena in uspešno implementirana nova strategija kombinacije klasičnih plemenilnih metod za hibridizacijo industrijskih aloploidov z visokim potencialom za proizvodnjo zaželenih hlapnih spojin (*S. cerevisiae* x *S. kudriavzevii*) z industrijskim sevom *S. cerevisiae*, mutantom s fenotipom za nizko proizvodnjo H_2S. Prvič so bili laboratorijsko razviti industrijski »food-grade« trojni aloploidni hibridi kvasovk. Novo razviti hibridi so industrijski sevi, ki izkazujejo dobro fermentacijsko kinetiko (Sliki 3 in 4, stran 34), proizvajajo visoke koncentracije zaželenih hlapnih snovi in nizke koncentracije nezaželenih spojin v vinski proizvodnji (Preglednica 3, stran 35). Na ta način je bila potrjena hipoteza A.

Medsebojni vplivi kontaminantov in starterskih kultur v enoloških razmerah

V vinogradništvu in vinarstvu se vedno več energije vlaga v varno pridelavo, kajti mednarodne smernice in potrošniki zahtevajo vedno bolj varno hrano, predvsem potrošniki pa želijo kakovost. Cilj direktiv mednarodnih organizacij je zmanjšati porabo pesticidov v pridelavi in obenem obdržati nizko stopnjo obolelosti vinske trte (EC Regulation, 2005). Za ohranjanje varnosti vin in izboljšanje njihovih senzoričnih lastnosti je potrebno do potankosti obvladovati vse dejavnike, ki vplivajo na te značilnosti.
V četrtem delu so bile analizirane interakcije med komplementarnimi kontaminanti, pesticidoma pirimetanil (Pyr) in fenheksamid (Fhx) in ohratoksinom A (OTA), ki je prisoten v večjih koncentracijah v grozdnih sokovih, pridobljenih iz grozdja, ki je manj škropljeno s pesticidi (Lo Curto in sod., 2004; Pardo in sod., 2005; Ribereau-Gayon in sod., 2006), z gensko različnimi sevi vinskih kvasovk v grozdnem soku. Namen četrtega dela raziskave je bil definirati, ali na kakovost vina bolj negativno vpliva intenzivno škropljenje s pesticidi ali infekcije grozdja in potencialna bolezenska stanja vinske trte.
Industrijski, gensko različni sevi, ki so bili izbrani, so bili sledeči: AWRI 0838 (*S.cerevisiae*), izolat komercialnega seva Lalvin 1118-EC, AWRI 1539 (*S. cerevisiae* x *S. kudriavzevii*), izolat komercialnega seva VIN7, AWRI 1640, mutant s fenotipom nizke proizvodnje H_2S PDM®

(Cordente in sod., 2009) ter AWRI 1810 ((*S. cerevisiae* x *S. kudriavzevii*) x (*S.cerevisiae*)), trojni hibrid, proizveden v tretjem delu raziskave.
Sev Lalvin 1118-EC smo uporabili kot referenčni sev skozi vse štiri raziskave.
Laboratorijske fermentacije so bile izvedene v grozdnem soku sorte Sauvignon (značilnosti so opisane na strani 41, Mediji in Reagenti). Izvedba fermentacijskih poskusov je bila enakega tipa kot v prvih dveh delih raziskave. Začetne koncentracije kontaminantov so bile izbrane na podlagi rezultatov iz prvih dveh delov raziskave, to so Pyr=10,0 mg/L; Fhx=10,0 mg/L in OTA=5,0 µg/L, koncentracije, pri katerih smo opazili interakcije v sintetičnem gojišču. Glede na to, da je medij drugačen (sterilno filtrirani grozdni sok) v primerjavi s prvima dvema deloma raziskave, YM (tekoča zmes kvasnega in sladnega ekstrakta z 18 % m/v glukoze) v fermentacijskih pogojih, so bile pričakovane razlike v rezultatih interakcij.
Od vsega začetka alkoholnih fermentacij je lahko opaziti, da imajo štirje industrijski sevi med seboj značilno različne fermentacijske kinetike: AWRI 1539 kot najhitrejši, AWRI 1640 najpočasnejši, ter AWRI 1810 in 0838 vmesnih hitrosti. Vsi so sposobni uspešno zaključiti kontrolno fermentacijo. Obenem se lahko iz fermentacijskih kinetik opazi negativen vpliv vseh kontaminantov. Fenheksamid je najmanj negativno vplival na vse seve, OTA je negativno vplival bolj, pirimetanil pa je vplival najbolj negativno in v primeru seva AWRI 1539 povzročil zaustavitev in nedokončano fermentacijo (Slika 1, stran 44; Sliki 2, 3, stran 45; Slika 4, stran 46). V primerjavi z opaženim v prvih dveh delih raziskave, v grozdnem soku OTA vpliva bolj negativno na fermentacijsko kinetiko vseh sevov v primerjavi s Fhx, ki je v mediju YM negativno vplival na kinetiko, OTA pa ne. V tej raziskavi je bilo prvič dokazano, da OTA in Fhx negativno vplivata na kinetiko gensko različnih industrijskih vinskih kvasovk med alkoholno fermentacijo. To, kar je bilo opaziti v prvih dveh delih raziskave, da so sevi s hitrejšo fermentacijsko kinetiko bolj občutljivi na kontaminante v sintetičnih medijih, lahko potrdimo tudi v četrtem delu, v grozdnem soku, kar kaže na manjšo fermentativno stabilnost le-teh v težkih industrijskih pogojih. Trend negativne intenzivnosti delovanja kontaminantov na posamezne seve je statistično značilno različen, kar potrjuje hipotezo E. Dodatno lahko ugotovimo, da medij, v katerem poteka fermentacija, značilno definira interakcije med sevi in kontaminanti, če primerjamo prva dva in četrti del raziskave.
Glede na to, da je bilo opaziti, da v sintetičnih medijih kontaminanti vplivajo na določene metabolne procese vinskih kvasovk, je bila v četrtem delu študija opravljena raziskava, kako le-ti delujejo na gensko različne industrijske seve med alkoholno fermentacijo in njihov vpliv na sestavo in kakovost vina.
Osnovna kemijska sestava oziroma osnovni tehnološki parametri po zaključeni alkoholni fermentaciji in hlapni fermentacijski produkti sevov definirajo senzorične lastnosti vina. Kvasovke pomembno vplivajo na vse omenjene faktorje, zato smo se odločili, da bomo preučili vpliv kontaminantov na obe sestavi (Fleet, 2003; Ribereau-Gayon in sod., 2006).
Splošni tehnološki parametri (Preglednica 2, stran 48) z izjemo koncentracije vinske kisline so spojine produkta primarnega metabolizma. Zaradi negativnega vpliva kontaminantov na fermentacijsko kinetiko je bil pričakovan negativen vpliv tudi na produkcijo le-teh. Rezultati za koncentracijo jabolčne kisline po zaključku alkoholne fermentacije kažejo na to, da ima sev AWRI 1640 najverjetneje neaktiven encim za degradacijo jabolčne kisline, to pa zaradi največjih koncentracij prisotne jabolčne kisline v vseh fermentacijskih poskusih (Mayer in Temperli, 1963; Radler, 1993). Neaktivnost encima je mogoče posledica nespecifične mutageneze, s katero je bil sev razvit (Cordente in sod., 2009). Poleg tega vmesna koncentracija jabolčne kisline v vinih, proizvedenih s sevom AWRI 1810, v primerjavi s koncentracijami v vinih, pridelanih z AWRI 1640 in AWRI 1539, kaže na mešano dedovanje hibrida. Podoben negativen efekt in sevna odvisnost koncentracij sta značilna tudi za citronsko,

mlečno in jantarno kislino, ki je še posebno pomembna za senzorične značilnosti vina (Ribereau-Gayon in sod., 2006).
Koncentracija ocetne kisline, katere prisotnost v visokih koncentracijah ima lahko negativen vpliv na senzorične lastnosti vina, je bila v primeru novo razvitega hibrida v tretjem delu naloge (AWRI 1810) zanimivo nizka. Presenetljivo ni bil opažen noben vpliv kontaminantov na produkcijo ocetne kisline pri kvasovkah, z izjemo AWRI 1640, kjer je bil opažen negativen efekt.
Na sintezo glicerola, komponente, ki ji pripisujejo telo vina, so vsi kontaminanti negativno vplivali v primeru vseh sevov, obenem pa sta se AWRI 0838 in 1810 izkazala kot manj občutljiva. Še več, AWRI 1810 je bil celo stimuliran za sintezo s strani pirimetanila. V tem primeru se kaže, da negativni učinek pirimetanila vpliva na podaljšano začetno fazo rasti in s tem povečano produkcijo glicerola (Ribereau-Gayon in sod., 2006), kar pričakovano zmanjša končno koncentracijo etanola. Proizvodnjo etanola je opaziti v trendu s fermentacijsko kinetiko za vse seve. Končna koncentracija etanola v vinu, proizvedenem z AWRI 1539 z dodanim pirimetanilom, je bila najnižja v trendu z nedokončano fermentacijo.
Fermentacijski produkti, ki jih proizvedejo vinske kvasovke v teku fermentacije, so determinirani s sevom starterske kulture in kemijsko sestavo medija (Fleet, 2003; Ribereau-Gayon in sod., 2006). Od interakcij med gensko različnimi kvasnimi sevi in kontaminanti smo pričakovali različne fermentacijske produkte.
Do sinteze etilnih estrov pride z encimatsko formacijo med prostimi alkoholi in acil-S-CoA (Shinohara in Watanabe, 1981) med alkoholno fermentacijo. Izkazalo se je, da je bil etil acetat, ki je poglavitni ester alkoholne fermentacije, proizveden v manjših koncentracijah ob prisotnosti kontaminantov pri vseh sevih. To je bilo tudi pričakovano v trendu zmanjšane koncentracije proizvedenega etanola in fermentacijske kinetike. Enak trend je bil ugotovljen tudi za etil heksanoat; oba omenjena estra sta izjemnega pomena za primerne senzorične lastnosti vina in oba sta bila proizvedena nad pragom zaznave v vinih (Robichaud in Noble, 1990; Garcia in sod., 2004). V nasprotju z omenjenima etil propanoat in etil butanoat nista bila proizvedena nad pragom zaznave, prisotnost kontaminantov pa je na njuno koncentracijo vplivala negativno. Prisotnost kontaminantov je pozitivno vplivala na proizvodnjo etil propanoata, z izjemo v primeru AWRI 1810, na katerega je OTA vplival delno negativno.
Ob prisotnosti kontaminantov se je proizvodnja etil 2-metilpropanoata, spojine z neželenim vonjem, povečala pri vseh sevih, kar še bolj negativno vpliva na senzorične lastnosti vina. Še dve spojini z neželenim vonjem sta bili okarakterizirani: 2-metilpropanoat in 3-metilpropanoat (Shinohara, 1985; Edwards in sod., 1990); zadnji je bil v vinu prisoten pod mejo detekcije, 2-metil propanoat pa je bil zaznan v minimalnih koncentracijah. Na koncentracijo le-tega je prisotnost kontaminantov vplivala negativno.
Vsi acetati so bili detektirani pod pragom senzorične zaznave (Garcia in sod., 2004). 2-feniletil acetat v mladem vinu daje floralni vonj po vijolicah in vrtnicah. Produkcija le-tega med alkoholno fermentacijo je bila sevno odvisna, obenem pa so na končno koncentracijo negativno vplivali kontaminanti, še posebno pirimetanil. Pirimetanil je najbolj negativno vplival na AWRI 0838 za produkcijo 2-feniletila v vinu. Po alkoholni fermentaciji ga nismo uspeli kvantificirati. Koncentracija ostalih analiziranih acetatov tj. 3-metilbutil acetata, 2-metilbutil acetata in 2-metilpropil acetata, se je pokazala kot sevno odvisna in negativno vplivana s strani vseh kontaminantov. Podoben trend je opaziti tudi v primeru heksil acetata, z izjemo OTA, ki ni statistično značilno vplival na sev AWRI 1539.
Višji alkoholi, ki jih proizvajajo kvasovke med alkoholno fermentacijo iz aminokislin in sladkorjev, njihovih prekurzorjev (Schreier, 1984; Giudici in sod., 1990; Dubois, 1994), pomembno vplivajo na senzorične lastnosti vin. Izoamilni alkoholi 2-metil butanol, 3-metil butanol in 2-metil propanol vinom dajejo vonj po sadju. Njihova proizvodnja se je pokazala

kot sevno odvisna; hibridi so bili največji proizvajalci. Kljub temu, da so vsi kontaminanti negativno vplivali na njihovo proizvodnjo, so bili vsi proizvedeni v večjih koncentracijah od praga zaznave v vinih (Oliva in sod., 1999).
Heksanol, ki daje vinom zeleno noto je bil edini, ki je bil v vseh vinih prisoten pod pragom senzorične zaznave. Proizvodnja heksanola se je med alkoholno fermentacijo izkazala kot sevno odvisna, AWRI 1640 pa je bil največji proizvajalec. Le pirimetanil je pomembno negativno vplival na proizvodnjo le-tega.
Ob zaželenih hlapnih snoveh smo se v raziskavi intenzivno ukvarjali z nastajanjem za vino nezaželene hlapne snovi, to je H_2S (Preglednica 1, stran 47).
Z razvojem AWRI 1810 v tretjem delu smo rešili problem proizvodnje H_2S med alkoholno fermentacijo, zato smo se v četrtem delu posvetili vplivom kontaminantov na proizvodnjo H_2S za gensko različne industrijske seve. Kot je bilo pričakovano, v poskusih z AWRI 1640 nismo zasledili proizvodnje H_2S. Za seva AWRI 0838 in 1539 se je pokazalo, da največ H_2S proizvedejo v poskusih, kjer ni bilo dodanega nobenega kontaminanta, torej kontaminanti negativno vplivajo na proizvodnjo H_2S v enakem trendu kot na kinetiko (Cabras in sod., 1995; Edwards in Bohlscheida, 2007). V primeru seva AWRI 1810, trojnega hibrida, pa sta pirimetanil in fenheksamid pomembno stimulirala proizvodnjo H_2S, ohratoksin A pa ni opazno vplival na ta sev. Rezultati kažejo, da imata pirimetanil in fenheksamid vpliv na kompleksen katabolni sistem za H_2S pri sevu AWRI 1810.

Iz rezultatov je razvidno, da vsi trije kontaminanti, pirimetanil in fenheksamid ter komplementarni ohratoksin A, pomembno vplivajo na kvasni metabolom med fermentacijskimi poskusi v grozdnem soku, oziroma v enoloških razmerah. Intenzivnost vpliva se je pokazala kot sevno in kontaminantno odvisna, kar potrjuje hipotezo F za fermentacijske poskuse v enoloških razmerah. To pomeni, da interakcije med gensko različnimi sevi in različnimi kontaminanti dajejo različne produkte. Obenem je opaziti, da noben kontaminant ne vpliva pozitivno na kemično sestavo vina, kar pomeni, da niti intenzivna raba pesticidov niti pridelava vina brez uporabe fitofarmacevtskih sredstev ni dobra praksa. Industrijski sevi, ki so bili raziskovani, vključeno z novo razvitim trojnim hibridom AWRI 1810, so pokazali stabilne fermentacijske lastnosti in sposobnost delovanja v težkih industrijskih pogojih z visokimi koncentracijami kontaminantov. Pri pridelavi vina so potrebni zelo pozorna kontrola praga infekcije vinske trte in natančno, upravljanje s fitofarmacevtskimi sredstvi, da lahko preprečimo bolezenska stanja trte in obenem zmanjšamo na minimum koncentracije pesticidov.
V zadnjem delu smo analizirali problem rezidualnih pesticidov in mikotoksinov na grozdju in možnost zmanjšanja njihove koncentracije v vinu v tehnološkem procesu proizvodnje. Kljub temu, da so kontaminanti delno odstranjeni v predfermentativnem delu procesa, lahko njihova koncentracija še vedno predstavlja nevarnost (Miller in sod., 1985; Tsiropoulos in sod., 2005; Kaushik in sod., 2008; Čuš in sod., 2010).
Ker je bilo v prvih dveh delih raziskave dokazano, da imajo vinski kvasni sevi sposobnost odstranjevanja kontaminantov v fermentativnih in stacionarnih fazah v sintetičnih medijih in da je edini mehanizem odstranjevanja adsorpcija, smo v četrtem delu raziskali interakcije v enoloških razmerah (Preglednica 4, stran 54).
Za poskuse je bil izbran enak fermentacijski načrt, vendar v različnem mediju v primerjavi s prvima dvema deloma, analizirali smo potencial za zmanjšanje koncentracije kontaminantov v dveh fazah, in sicer po dokončanju alkoholne fermentacije oziroma njeni zaustavitvi in po sedmih dnevih postfermentacijskega podaljšanega kontakta s kvasovkami (»battonage«). V prvem delu raziskave je bilo dokazano in prej potrjeno (Nunez in sod., 2007), da se iz celičnih sten kvasovk sprosti glavni del manoproteinov, glavnih vezalnih agensov za kontaminante,

prav v postfermentativni fazi in definira totalno kapaciteto zmanjšanja koncentracije kontaminantov za sev. Rezultati, ki so bili pridobljeni za vse kontaminante, potrjujejo, kar je bilo prikazano pri fermentacijskih poskusih v sintetičnih medijih, in sicer, da vrsta medija vpliva na stopnjo zmanjšanja koncentracije kontaminanta. Okolje intenzivno vpliva na fizikalno-kemijske mehanizme odstranjevanja kontaminantov (Cabras in sod., 1999; Caridi, 2006; Caridi in sod., 2006; Angioni in sod., 2007). AWRI 0838, ki je bil uporabljen kot referenčni sev, kaže značilno različen potencial za zmanjšanje koncentracije kontaminantov v dveh medijih. V enoloških pogojih se pokaže, da pride do pomembnega zmanjšanja koncentracije kontaminantov že po zaključku alkoholne fermentacije in ne samo med postfermentacijskim podaljšanem kontaktu, kot se je pokazalo v sintetičnem mediju. Zmanjšanje koncentracije kontaminantov v obeh fazah v enoloških razmerah potrjuje hipotezi B in C, ker je lahko opaziti, da je v dveh fazah stopnja zmanjšanja značilno različna. Ker je bila v primerjavi s fermentacijskimi poskusi v sintetičnem mediju v poskusih v enoloških razmerah prisotna visoka stopnja genske raznolikosti, smo dokazali hipotezo D, ki smo jo v sintetičnem mediju dokazali le delno. Genom sevov močno določa sposobnost zmanjšanja kontaminantov, ker vpliva na občutljivost seva, kemijsko sestavo celičnih sten in proizvodnjo manoproteinov, obliko in razmerje volumen/površina ter dinamiko avtolize (Cabras in sod., 1999; Martínez-Rodriguez in sod., 2001; Martínez-Rodríguez in sod., 2001; Cabras in sod., 2003; Bejaoui in sod., 2004; Caridi, 2006; Mazauric inSalmon, 2006; Caridi, 2007; Nunez in sod., 2007; Čus in Raspor, 2008; Gonzalez-Ramos in sod., 2008).
Sposobnost zmanjšanja koncentracije pirimetanila s strani seva AWRI 1539 je klasičen primer, ko je občutljivost seva na kontaminant visoka in nesposobnost le-tega, produkcije visokih koncentracij biomase onemogoča večjo stopnjo zmanjšanja koncentracije kontaminanta. Obenem AWRI 1810 tudi tokrat kaže na mešano dedovanje, kajti sposobnost za zmanjšanje koncentracij pirimetanila je vmesna v primerjavi s starševskima sevoma.
Stopnja zmanjšanja ostalih kontaminantov se kaže kot sevno in kontaminantno odvisna (Preglednica 4, stran 54).

SKLEPI

Iz dobljenih rezultatov izhajajo naslednje ugotovitve:

- Prikazana strategija kombinacije klasičnih plemenilnih tehnik, ki je bila uporabljena za izboljšanje industrijskih sevov, je omogočila, da so bili prvič razviti trojni aloploidni hibridi s fenotipom nizkega potenciala proizvodnje H_2S, intenzivno proizvodnjo zaželenih hlapnih snovi in stabilno fermentacijsko kinetiko.

- Industrijski in avtohtoni sevi vinskih kvasovk so sposobni zmanjšati koncentracijo pirimetanila, fenheksamida in ohratoksina A v sintetičnih medijih in grozdnem soku, v stacionarnih in fermentativnih pogojih; mehanizem za odstranjevanje kontaminantov iz medijev implicira le adsorpcijo in ne metabolno degradacijo s strani kvasne biomase.

- Potencial za odstranjevanje kontaminantov metabolno aktivne biomase je statistično značilno različen v fermentativni fazi od metabolno aktivne biomase po končani alkoholni fermentaciji, odvisen je od seva kvasovke, tipa kontaminanta in medija, v katerem se fermentacija odvija.

- Visoke koncentracije ohratoksina A, pirimetanila in fenheksamida negativno vplivajo na fermentacijsko kinetiko gensko različnih industrijskih sevov, od najbolj proti

najmanj negativnemu si sledijo: pirimetanil, ohratoksin A in fenheksamid. Sevi se različno odzivajo na prisotnost različnih kontaminantov, vendar ima na te interakcije močan vpliv medij, v katerem se interakcija odvije.

- Visoke koncentracije ohratoksina A, pirimetanila in fenheksamida so sposobne vplivati na ekso-metabolom gensko različnih industrijskih sevov med alkoholno fermentacijo; interakcije med različnimi sevi – genetskimi ozadji in različnimi kontaminanti dajejo različneproizvode, vendar ima tudi v tem primeru medij pomemben vpliv.

Poleg glavnih sklepov smo dodatno ugotovili, da

- se je AWRI 1640 izkazal kot odlično orodje za uporabo v plemenilnih strategijah za zmanjševanje proizvodnje H_2S med alkoholno fermentacijo pri industrijskih sevih;

- je točkovna mutacija v genu *MET10* (G176A) pri sevu AWRI 1640 dominantna;

- je pri vinskih sevih z neaktivnim encimom sulfit reduktaza možno ohraniti nizko proizvodnjo SO_2 med alkoholno fermentacijo;

- imajo novi trojni aloploidni hibridi majhen potencial za proizvodnjo hlapnih kislin;

- je bil v sterilnem grozdnem soku ugotovljen večji potencial kvasnih sevov za odstranjevanje kontaminantov iz medija v primerjavi s potencialom v YM (tekoča zmes kvasnega in sladnega ekstrakta z 18 % m/v glukoze); po zaključku alkoholne fermentacije ni bilo zaslediti pomembnega zmanjšanja koncentracije kontaminantov v gojišču YM, slednje se je zgodilo le v podaljšani postfermentacijski fazi;

- je bila v vseh medijih ugotovljena večja stopnja varnosti po zaključku alkoholne fermentacije, in to zaradi odstranitve kontaminantov s strani kvasne biomase;

- je vpliv kontaminantov na kinetiko in ekso-metabolom kvasovk intenzivneje negativen v grozdnem soku v primerjavi s sintetičnim medijem;

- je negativni efekt kontaminantov bolj intenziven za seve s hitrejšo fermentacijsko kinetiko;

- prisotnost vseh treh kontaminantov v grozdnem soku negativno vpliva na senzorične lastnosti vina, kar pomeni, da vsi trije kontaminanti navkljub komplementarnosti negativno vplivajo na kakovost vina;

- optimalno kakovost vina lahko dosežemo z industrijskimi sevi z dobrimi enološkimi lastnostmi in s tako najmanjšo možno uporabo pesticidov med vegetacijo, ki še vedno prepreči bolezenska stanja trte in stranske produkte le-teh, kajti navkljub komplementarnosti fitofarmacevtskih sredstev z ohratoksinom A vsi trije kontaminanti negativno vplivajo na kakovost vina, na njegovo varnost in na njegove senzorične lastnosti.

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