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REVIEW

Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking

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Abstract Biotechnology as applied to winemaking includes several aspects of the fermentation industry, such as monitoring of microbial populations, use of selected starter cultures, and control of undesired yeasts. Over the last few decades, the control of microorganisms using biotechnological approaches has become of increasing importance in the winemaking field. The profusion of selected starter strains has allowed more extensive use of inoculated fermentations, with a consequent improvement in the control of fermentation combined with the use of new biotechnological processes in winemaking. As a consequence of this re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking over the last few years, several studies have evaluated the use of controlled mixed fermentations using *Saccharomyces* and different, non-*Saccharomyces*, yeast species that are a part of the winemaking environment. In this context, mixed fermentations using controlled inoculations of *Saccharomyces cerevisiae* starter cultures and non-*Saccharomyces* yeasts represent a practical way towards improving wine complexity and enhancing specific characteristics of a wine. Another trait in the use of non-*Saccharomyces* yeasts in winemaking relates to the control of spoilage microorganisms. Indeed, more strict control of undesirable yeasts is required during the various phases of wine fermentation. Moreover, there is now increasing interest in the use of natural antimicrobial agents in foods and, in this context, killer yeasts might have important roles in the control of spontaneous and/or spoilage microflora. Thus, killer toxins appear to represent an attractive solution for use as antimicrobial agents, to partially, or even completely, substitution for chemical agent use even if application costs could limit their use in winemaking.

Keywords Multistarter fermentation · Killer yeast · Alcoholic fermentation · Biotechnology approach

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Introduction

Saccharomyces cerevisiae plays a fundamental role in the anaerobic transformation of grape must into wine, where the fermenting medium can be inoculated with selected commercial yeast strains or left to ferment with the natural flora present on the grapes. In both cases, *S. cerevisiae* governs fermentation (Bisson 2004).

Biotechnological approaches to winemaking are used in several aspects of the fermentation industry, such as formulation and use of selected starters cultures, monitoring of microbial populations, and control of spoilage yeasts (Reed and Nagodawithana 1988; Pretorius 2000). Over the last few decades, the improved use and control of microorganisms through biotechnological approaches has become increasingly important in winemaking. The profusion of selected starter cultures as active dry yeast (ADY) forms has allowed more widespread use of inoculated fermentation, with consequent improvements in control of the fermentation process, which can be combined with new biotechnological processes in winemaking.

In recent years, re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking has resulted in

several studies that have looked at the use of controlled mixed fermentations using *Saccharomyces* along with non-*Saccharomyces* yeast species from the winemaking environment (Lema et al. 1996; Ciani and Maccarelli 1998; Heard 1999; Jolly et al. 2003; Ciani et al. 2010). These have demonstrated that mixed fermentations using controlled inoculations of *S. cerevisiae* starter cultures and non-*Saccharomyces* yeasts represent a feasible way towards improving wine complexity and enhancing particular and specific characteristics of a wine. Indeed, fermentations carried out using mixed and controlled inocula can improve the quality of the final product, and can assure both a more standard fermentation process and an enhancement of the analytical composition of a wine, by taking advantage of several metabolic pathways of non-*Saccharomyces* yeast strains.

Another possible biotechnological use of non-*Saccharomyces* yeasts in winemaking relates to the control of undesired microorganisms. Widely different yeasts species can participate in the various stages of winemaking, which can result in an undesired organoleptic profile of the final product. In this context, there is the need for more strict control of potential spoilage microorganisms during the various stages of wine production: here, the natural antimicrobial agents that can be produced by non-*Saccharomyces* yeasts can play important roles in the control of spontaneous and/or spoilage microflora.

Influence of non-*Saccharomyces* yeasts in mixed fermentations

It is well established that *S. cerevisiae* is the main microorganism involved in alcoholic fermentation of grape must. However, winemaking is a non-sterile process, so the rich composition and the analytical complexity of the grape must can allow the development of a broad number of yeast species that belong to various non-*Saccharomyces* genera. In the past, these yeasts were considered as undesired or spoilage yeasts, as they can confer a negative sensorial profile to the final wine (Amerine and Cruess 1960; Ribereau-Gayon and Peynaud 1960). Indeed, grape musts inoculated with pure cultures of non-*Saccharomyces* yeasts have shown to produce several metabolites at unfavourable levels, including acetic acid, acetoin, ethyl acetate and acetaldehyde; this would thus exclude the use of such non-*Saccharomyces* yeasts in selected starter cultures. However, when non-*Saccharomyces* yeasts are cultivated as mixed fermentations with the *S. cerevisiae* strain, their negative metabolic activities might not be expressed, or could be modified by the metabolic activity of the starter culture of the *S. cerevisiae*. In this context, in recent years, several studies have proposed the use of selected non-*Saccharomyces* yeasts together with an *S. cerevisiae* starter strain during wine fermentation, highlighting the positive results that can be produced with mixed fermentations (Zironi et al. 1993; Lema et al. 1996; Romano et al. 1997; Ciani and Maccarelli 1998; Egli et al. 1998; Henick-Kling et al. 1998; Rojas et al. 2001; Fleet 2003; Jolly et al. 2003; Ciani et al. 2006; Kim et al. 2008).

Possible synergistic interactions between different yeast species in mixed fermentations represent a valuable instrument for innovative fermentation technologies (Mendoza et al. 2007; Fleet 2008; Ciani et al. 2010). The influence of multistarter fermentation practices on final wine composition and on growth and death rates of the *S. cerevisiae* and non-*Saccharomyces* strains has been investigated. Mora et al. (1990) reported on the growth of *Kluyveromyces* (*Lachancea*) *thermotolerans* and *S. cerevisiae* species in sequential cultures, highlighting the significant reduction in acetic acid production during this mixed fermentation. Other studies have been carried out with the aim of de-acidifying the grape must or wine through malic acid degradation using mixed fermentations of *Schizosaccharomyces pombe* and *S. cerevisiae* (Snow and Gallender 1979; Magyar and Panyik 1989; Yokotsuka et al. 1993). To enhance the glycerol content in wine, *Starmerella bombicola* (formerly *Candida stellata*) has been proposed for mixed fermentations with *S. cerevisiae* starter cultures. Several studies have shown that the inoculation of grape-must fermentations with pure cultures of *Starmerella bombicola* can result in the production of high concentrations of acetaldehyde and acetoin (Ciani and Ferraro 1996, 1998; Ferraro et al. 2000). However, different results were obtained in the fermentation of grape musts with mixed fermentations of *Starmerella bombicola* and *S. cerevisiae* starter strains. In this latter case, the analytical and organoleptic profile of the wine was improved without any negative analytical profiles. Indeed, in comparison with *S. cerevisiae*-inoculated fermentations, the use of mixed or sequential inoculations, continuous fermentation, and immobilised cells can provide: (1) complementary consumption of glucose and fructose; (2) improved glycerol and succinic acid contents; and (3) no increases in acetaldehyde and acetoin contents, due to the presence of the *Starmerella bombicola* (i.e. exchange of acetaldehyde between the two species without any increment in its levels).

The practice of multistarter fermentation can also be used to improve the complexity of the final aroma and flavour of a wine. Garcia et al. (2002) studied the influence of mixed cultures of *Debaryomyces vanriji* and *S. cerevisiae* for improvement of geraniol production, due to the high levels of β-glucosidase activity showed by this non-*Saccharomyces* strain.

Recent investigations using multistarter fermentations (mixed and sequential inocula) with starter strains of

Torulaspora delbrueckii and *Kluyveromyces* (*Lachancea*) *thermotolerans* together with *S. cerevisiae* have shown that the relevant detectable effects included: (1) consistent reduction in acetic-acid content (*T. delbrueckii*); and (2) reduction in acetaldehyde concentration and increase in titratable acidity (*K. thermotolerans*) (Ciani et al. 2006; Bely et al. 2008).

Yeasts belonging to the *Hanseniaspora/ Kloeckera* genera have long been considered as spoilage yeasts, particularly during the first stage of fermentation, due to their ability to produce undesired compounds, such as acetic acid and ethyl acetate. Mixed fermentation trials with apiculate yeasts and *S. cerevisiae* starter cultures have shown increases in isoamyl acetate in the presence of *H. uvarum* (Moreira et al. 2008), while use of *Hanseniaspora osmophila* provides improvements in 2-phenylethyl acetate production (Viana et al. 2009). As reported by Kurita (2008), inoculated mixed fermentations using *Pichia anomala* resulted in positive enhancement of isoamyl acetate. Another strain belonging to the *Pichia kluyveri* species has been used in mixed fermentations with *S. cerevisiae*, producing an improvement in the varietal thiols (Anfang et al. 2009).

The main effects of multistarter fermentations, as compared with inoculation with pure *S. cerevisiae* cultures, are given in Table 1.

Non-*Saccharomyces* yeasts in the control of undesirable yeasts

Another aspect of the use of non-*Saccharomyces* yeasts in winemaking relates to the control of undesired microorganisms. Stricter control of potential spoilage microorganisms during the various stages of wine production is needed. Indeed, a large number of yeasts can participate in the various phases of the fermentation process, sometimes resulting in undesired organoleptic characters in the final product.

There is now increasing interest in the use of natural antimicrobial agents in foods and, in this context, yeast killer systems could play important roles in the control of spontaneous and/or spoilage microflora. Killer toxins appear to represent an interesting solution as antimicrobial agents, for the partial or complete substitution of the use of chemical agents. Screening to determine the occurrence of killer yeasts in winemaking environments has formed part of several studies, and these have shown the widespread presence of killer and neutral phenotypes in wines, and on cellar surfaces and winery equipment, while the sensitive yeasts were recovered only occasionally (Rosini et al. 1988; Rosini and Ciani 1988; Musmanno et al. 1999).

Killer yeast strains have been isolated from various oenological sources, including grape berries, grape musts, and wines, both during fermentation and as the final product (Heard and Fleet 1987). Other studies have been carried out to determine the significance of such killer activities in winemaking (Vagnoli et al. 1993; Ramon-Portugal et al. 1998; Sangorrín et al. 2007). The widespread occurrence of these killer yeasts has been demonstrated in strains isolated from vineyards and wineries in different regions of the World, such as Europe, Australia, Argentina (Patagonia region) and South Africa (Petering et al. 1991).

Over the last two decades, particular attention has been directed towards the selection of starter strains of *S. cerevisiae* species that have these killer characteristics (van Vuuren and Jacobs 1992). However, the main limitation to the application of the killer toxins that these *S. cerevisiae* yeasts produce resides in their narrow anti-yeast spectra, which is restricted to sensitive *Saccharomyces* strains. Thus, these killer toxins do not affect wild yeasts, such as those belonging to the genera *Hanseniaspora/Kloeckera*, *Pichia*, *Brettanomyces*, *Zygosaccharomyces* and *Saccharomycodes*, which represent the main targets of the antimicrobial agents used in winemaking. Due to this absence of killing action towards these spoilage yeasts, the killer activity can be considered as an additional character for *S. cerevisiae* starter-strain selection (Mannazzu et al. 2002).

However, species of *Hanseniaspora/Kloeckera*, *Candida*, *Hansenula* and *Pichia* can establish significant growth during wine fermentations (Fleet 1990). Consequently, the potential for the production of killer toxins that affect *S. cerevisiae* by these species of non-*Saccharomyces* yeasts becomes important, as does the susceptibility of these yeasts to toxins produced by *S. cerevisiae*. Thus, in theory, selected killer yeast strains can also be used as the inoculated strain, so as to suppress the growth of undesirable wild strains during grape-juice fermentation.

The control of indigenous microflora at prefermentative stages and during fermentation is now generally carried out through the addition to the must of starter cultures. These have known and desired genetic characteristics at the pre-fermentation stage, and are coupled with the use of sulphur dioxide, to combat contaminating wild yeasts (Ribereau-Gayon and Peynaud 1960). Sulphur dioxide has a long history of use as a yeast preserver in the manufacture of alcoholic beverages, especially of wine, although this antiseptic agent can have adverse effects on the respiratory systems of humans and animals, and can damage vegetation. In this context, the use of a killer toxin as a control agent towards non-*Saccharomyces* yeasts such as apiculate yeasts at the prefermentative stage and during fermentation of grape must should be further encouraged so as to reduce, or even eliminate, the use of sulphur dioxide. Another interesting potential application of the killer character in winemaking is seen for killer toxins that are active against the *Dekkera/Brettanomyces* spoilage

Table 1 Fermentation behaviour of non-*Saccharomyces* and *Saccharomyces cerevisiae* strains in multistarter inocula

Non-*Saccharomyces* yeast species	Characteristic behaviour of pure culture	Effects produced by mixed fermentation with *S. cerevisiae*, compared with pure *S. cerevisiae* culture
Starmerella bombicola	Fructophilic yeast	Combined consumption of reduced sugars (improved consumption)
	High glycerol producer	Increase in glycerol production
	High succinic acid producer	Increase in succinic acid production
	High acetaldehyde producer	No increase (combined consumption)
	High acetoin producer	No increase (combined consumption)
	Low ethanol yield	Reduction in final ethanol concentration
Kluyveromyces thermotolerans	Low acetaldehyde producer	Reduction in final acetaldehyde formation
	Lactic acid producer (some strains)	Increase in titratable acidity
Hanseniaspora uvarum	High acetic acid producer	No increase in acetic acid production
	High ethyl acetate producer	Slight increase in ethyl acetate production (strong reduction in comparison with pure culture)
Torulaspora delbrueckii	Low acetic acid producer	Reduction in acetic acid production
Hanseniaspora osmophila	High 2-phenyl ethyl acetate producer	Increase in 2-phenyl ethyl acetate
Pichia anomala	High producer of isoamyl acetate (EAHase)	Increase in isoamyl acetate production
Pichia kluyveri	High producer of 3-mercaptohexyl acetate	Increase in thiols content
Debaryomyces variji	High level of β-glucosidase activity	Increase in terpenols content
Schizosaccharomyces spp.	High rate of malic acid degradation	Reduction in titratable acidity

yeasts that represent a major problem in the wine industry (Sponholz 1993). These yeasts are not commonly found on the surface of the grape berry or in the must, although they can increase in wines during wine ageing in wooden barrels (Ibeas et al. 1996).

Killer character in the control of apiculate yeasts

Recently, it has been shown that the yeast *Kluyveromyces phaffii* (now reclassified as *Tetrapisispora phaffii*; Ueda-Nishimura and Mikata 1999) can produce a killer toxin (the

Table 2 Principal traits of the killer activity of *Tetrapisispora phaffii* (Kpkt)

Investigated traits of *T. phaffii*	Results	Methodology
Diffusion among *Hanseniaspora* strains	94.9% (on 298 assayed isolates from wine environment)	Streak plate diffusion assay
Range of activity	pH range of activity from 2.8 to 5.0; optimal temperature of 20°C (max 35°C)	Well test assay in malt agar medium
Biochemical and physiological characterisation	Molecular mass: 33,070 Da	Mass spectrometry (MALDI-TOF-MS)
	Glycoprotein with glucidic portion (10%)	Endoglycosidase-H treatment
	Receptor site: β-1,3 branched glucans	Binding of KpKt to cell-wall polysaccharides
	NH_2-terminal sequence: 93% identity with β-1,3-glucanase of *S. cerevisiae*, and 80% identity with β-1,3-glucantransferase of *Candida albicans*	15 cycles of NH_2-sequence analysis
	β-glucanase activity (0.830 μmol mg^{-1} min^{-1})	Enzymatic assay
Mode of action	Disruption of cell-wall integrity mediated by a highly specific β-glucanase activity	Confocal, laser-scanning electron and atomic force microscopy.
		Enzymatic activities
		Endoglycosidase-H treatments
		Flow-cytometry analyses
Activity in winemaking	Control of growth and metabolic activity of *H. uvarum*	Microfermentation trials using free and immobilized *T. phaffii* cells
	Significant inhibition halo (from 12 to 15 mm) during fermentation	Well test assay in malt agar medium using concentrated wine
	Control of analytical composition of wines	Assessment of analytical profiles of wines

zymocin KpKt) that is active on wine yeasts that can contaminant the prefermentation stage (Ciani and Fatichenti 2001). Indeed, the KpKt toxin produced by *Kluyveromyces phaffii* DBVPG 6076 can be used as a biological agent against apiculate yeasts, which are usually present in the freshly pressed grape juice and during the first stage of alcoholic fermentation. KpKt has a wide anti-*Hansenispora*/*Kloeckera* activity under winemaking conditions, and therefore it is of particular interest for its application as an antimicrobial agent in the wine industry. Indeed, like most proteinaceous killer toxins, KpKt is active in the pH range of 3.0–5.0 and at temperatures lower than 40°C. Moreover, its fungicidal or fungistatic effects depend on its concentration, and the inhibitory activity of the KpKt killer toxin in grape juice has been shown to be comparable to that of sulphur dioxide (Ciani and Fatichenti 2001).

Further characterization of KpKt has shown that this toxin is a glycosylated protein with a molecular mass of 33.07 kDa, and with an NH_2-terminal sequence that does not show any similarities to that of other known killer toxins (Shimizu 1993; Magliani et al. 1997). According to a BLAST analysis of the 15 amino acids of its NH_2-terminal sequence, KpKt is strikingly similar to β-1,3-glucanase of *S. cerevisiae* and β-1,3-glucan transferase of *Candida albicans*. Indeed, competitive inhibition of KpKt activity by cell-wall polysaccharides has shown that the cytocidal action of KpKt is prevented by glucan β-1,3-branched and β-1,6-branched glucans (Comitini et al. 2004a).

In a recent study, Comitini et al. (2009b) demonstrated that KpKt induces ultrastructural modifications in the cell wall of sensitive yeast strain, effects that are mediated by the glucidic portion of this toxin. All of the data obtained indicate that the mode of action of KpKt involves disruption of cell-wall integrity. The principal traits of the killer activity of Kpkt are shown in Table 2.The validity of the zymocidial activity of KpKt on *H. uvarum* during fermentation has been investigated in grape must microfermentation trials with inoculated free and immobilized *T. phaffii* cells (Comitini and Ciani 2010). The microbial evolution and fermentation profiles of the wines were evaluated to determine the effects of KpKt on apiculate yeasts, and the results were compared with those of sulphur dioxide addition. The fungicidal activity of KpKt against *H. uvarum* was stable for at least 14 days in the wine, resulting in active control of the proliferation of apiculate yeasts. At the same time, the analytical composition of these wines with the inoculum of *T. phaffii* immobilized cells did not differ from the wines with sulphur dioxide added. In contrast, in wines without control of apiculate yeasts, there was an increase in ethyl acetate. These preliminary findings thus show that *T. phaffii* is an excellent candidate for the biological control

Table 3 Principal characteristics of anti-*Brettanomyces* killer toxins of *Kluyveromyces wickerhamii* (KwKt), *Pichia anomala* (Pikt) and *Pichia membranifaciens* (PMKT2)

Investigated traits of anti-*Brettanomyces* killer toxins	Results
Screening	KwKt: 100% (on 15 assayed isolates from wine environment)
	Pikt: 100% (on 15 assayed isolates from wine environment)
	PMKT2: 80% (on 20 assayed isolates from wine environment)
Molecular mass	KwKt: 75 kDa
	Pikt: 8 kDa
	PMKT2: 30 kDa
Specific receptor sites	KwKt: pustulans
	Pikt: β-1,6-glucans
	PMKT2: mannoproteins
Range of action	KwKt: pH range of activity from 3.8 to 4.6; optimal temperature of 20°C (max 25°C)
	Pikt: Optimal pH 4.4; optimal temperature of 25°C (max 35°C)
	PMKT2: optimal pH 4.5; optimal temperature below 20°C
Activity in winemaking	KwKt and Pikt: Killer/sensitive ratio of 1:10 delays growth of sensitive cells; killer/sensitive ratio of 1:1 completely inhibits growth;
	Different metabolic activity: reduced ethyl phenols production in wine fermented with killer strains, in comparison with control without killer yeasts.
	PMKT2: killer activity under winemaking physico-chemical conditions and compatible in terms of competence with the fermentative process (PMKT2 does not interfere with *S. cerevisiae* fermentation)

of undesired proliferation of apiculate yeasts during the first stages of fermentation, and that *T. phaffii* cells in an immobilized form can be used as a biocontrol agent to avoid, or at least reduce, the use of sulphur dioxide.

Killer yeasts active against *Dekkera/Brettanomyces* spoilage yeasts

Dekkera/Brettanomyces yeasts are recognised as common contaminants in wine, and can produce strong off-flavours. Some key periods are important for "managing brett". The risks for these "brett"-note deviations are not only real at the beginning of the fermentation for the indigenous *Brettanomyces*, but also during other key or risky moments in the winemaking process (e.g. end of fermentation, maturating, bottling).

Considering that sulphur dioxide is an important consumer issue, and that it can reduce or block the maturation of wines, that wine filtration can affect the body and viscosity of wines, and that steam treatments for barrels can damage the wooden barrels, microbiological alternatives using bioactive compounds to help to limit the growth of *Brettanomyces* should be well accepted by winemakers.

In this context, the exploration of killer yeasts as producers of the mycocins that can counteract the activities of these undesired microorganisms in wine appears of particular interest. Recently, two killer yeasts, *Kluyveromyces wickerhamii* and *Pichia anomala*, were shown to secrete two toxins, KwKt and Pikt, respectively, that are active against *Dekkera/Brettanomyces* spoilage yeasts (Comitini et al. 2004b). These mycocins can counteract the activities of these undesired microorganisms in wine. This preliminary characterisation showed that KwKt and Pikt are stable within a pH range from 3 to 5 and at temperatures below 35°C, and that they maintain their killer activities for at least 10 days in wine. Further characterisation of Pikt has indicated that this toxin is a ubiquitin-like protein with an apparent molecular mass of 8 kDa (De Ingeniis et al. 2009). A more recent study (Comitini et al. 2009a) carried out in grape must and wine showed that *K. wickerhamii* and *P. anomala* can indeed control *Brettanomyces/Dekkera* spoilage yeasts. Preliminary results showed that a ratio of killer yeast to sensitive yeast (killer/sensitive ratio) of 1:10 delays the growth of *D. bruxellensis* cells, while a killer/sensitive ratio of 1:1 completely inhibits the growth of this sensitive strain. These preliminary findings indicate that these killer yeasts can indeed control both growth and metabolic activity of these sensitive spoilage yeasts. Indeed, there was a reduction in the levels of the unpleasant molecules that are considered to be the most involved in "brett" notes in wines, i.e. the ethyl phenols (Comitini et al. 2004b).

Recently, Santos et al. (2009) investigated a new killer toxin that is produced by *Pichia membranifaciens* (PMKT2), that showed activity against *Brettanomyces bruxellensis*. Biochemical characterization of the purified PMKT2 showed an apparent molecular mass of 30 kDa, an optimal pH of 4.5, and the best killer action at temperatures up to 20°C. Also of note, PMKT2 inhibited *B. bruxellensis* while *S. cerevisiae* was fully resistant, indicating that PMKT2 can be used in wine fermentations to avoid the development of the spoilage yeast *B. bruxellensis* without deleterious effects on the *S. cerevisiae* fermentative strain. The killer activity of PMKT2 was evaluated also under simulated winemaking conditions, and the results obtained in small-scale fermentations showed that PMKT2 can indeed inhibit *B. bruxellensis* under these conditions. The principal characteristics of anti-*Brettanomyces* killer toxins are showed in Table 3.

In conclusion, zymocins produced by non-*Saccharomyces* yeasts appear to have promising applications in winemaking for the control of *Dekkera/Brettanomyces* spoilage yeasts. Considering that *Dekkera/Brettanomyces* yeasts cause heavy economic losses in winemaking, these natural antimicrobial compounds appear to be suitable tools for the control of this contamination risk.

References

Amerine MA, Cruess WV (1960) The technology of winemaking. AVI, Connecticut

Anfang N, Brajkovich M, Goddard MR (2009) Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Savignon Blanc. Aust J Grape Wine Res 15:1–8

Bely M, Stoeckle P, de Masnuef-Pomare I, Dubourdieu D (2008) Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. Int J Food Microbiol 122:312–320

Bisson L (2004) The biotechnology of wine yeast. Food Biotechnol 18:63–96

Ciani M, Fatichenti F (2001) Killer toxin of *Kluyveromyces phaffii* DBVPG 6076 as a biopreservative agent to control apiculate wine yeasts. Appl Environ Microbiol 67:3058–3063

Ciani M, Ferraro L (1996) Enhanced glycerol content in wines made with immobilized *Candida stellata* cells. Appl Environ Microbiol 62:128–132

Ciani M, Ferraro L (1998) Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improbe the quality of wines. J Appl Microbiol 85:247–254

Ciani M, Maccarelli F (1998) Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. World J Microbiol Biotechnol 14:199–203

Ciani M, Beco L, Comitini F (2006) Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. Int J Food Microbiol 108:239–245

Ciani M, Comitini F, Mannazzu I, Domizio P (2010) Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. FEMS Yeast Res 10:123–133

Comitini F, Ciani M (2010) The zymocidial activity of *Tetrapisispora phaffii* in the control of *Hanseniaspora uvarum* during the early stages of winemaking. Lett Appl Microbiol 50:50–56

Comitini F, Di Pietro N, Zacchi L, Mannazzu I, Ciani M (2004a) *Kluyveromyces phaffii* killer toxin active against wine spoilage yeasts: purification and characterization. Microbiology 150:2535–2541

Comitini F, De Ingeniis J, Pepe L, Mannazzu I, Ciani M (2004b) *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera/Brettanomyces* spoilage yeasts. FEMS Microbiol Lett 238:235–240

Comitini F, Gobbi M, Languet P, Ciani M (2009a) Zymocidial activity of two killer yeasts to keep under control the development of *Brettanomyces/Dekkera* in winemaking. Book abstracts 2nd International Symposium "Micro Safety Wine" Martina Franca (TA), Italy, 18-20 November, p. 22. http://www.mycotox-society.org/files/news/23_Atti_Microsafetywine_20092.pdf

Comitini F, Mannazzu I, Ciani M (2009b) *Tetrapisispora phaffii* killer toxin is a highly specific beta-glucanase that disrupts the integrity of the yeast cell wall. Microb Cell Fact 8:55. doi:10.1186/1475-2859-8-55

De Ingeniis J, Raffaelli N, Ciani M, Mannazzu I (2009) *Pichia anomala* DBVPG 3003 secretes a ubiquitin-like protein that has antimicrobial activity. Appl Environ Microbiol 75:1129–1134

Egli CM, Ediger WD, Mitrakul CM, Henick-Kling T (1998) Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and chardonnay wines. J Appl Microbiol 85:779–789

Ferraro L, Fatichenti F, Ciani M (2000) Pilot scale vinification process by immobilised *Candida stellata* and *Saccharomyces cerevisiae*. Process Biochem 35:1125–1129

Fleet GH (1990) Growth of yeasts during wine fermentation. J Wine Res 1:211–224

Fleet GH (2003) Yeast interactions and wine flavour. Int J Food Microbiol 86:11–22

Fleet GH (2008) Wine yeasts for the future. FEMS Yeast Res 8:979–995

Garcia A, Carcel C, Dalau L, Samson A, Aguera E, Agosin E, Gunata Z (2002) Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomyces cerevisiae* on the volatiles in a Muscat wine. J Food Sci 67:1138–1143

Heard GM (1999) Novel yeasts in winemaking—looking to the future. Food Aust 51:347–352

Heard GM, Fleet GH (1987) Occurrence and growth of killer yeasts during wine fermentation. Appl Environ Microbiol 53:2171–2174

Henick-Kling T, Ediger W, Daniel P, Monk P (1998) Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J Appl Microbiol 84:865–876

Ibeas JI, Lozano I, Perdigones F, Jimenez J (1996) Detection of *Dekkera-Brettanomyces* strains in sherry by a nested PCR method. Appl Environ Microbiol 62:998–1003

Jolly NP, Augustyn OPH, Pretorius IS (2003) The use of *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine. S Afr J Enol Vitic 24:63–69

Kim DH, Hong YA, Park HD (2008) Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduces the malic content in wine. Biotechnol Lett 30:1633–1638

Kurita O (2008) Increase of acetate ester-hydrolysing esterase activity in mixed cultures of *Saccharomyces cerevisiae* and *Pichia anomala*. J Appl Microbiol 104:1051–1058

Lema C, Garcia-Jares C, Orriols I, Angulo L (1996) Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some compounds of Albarino wine aroma. Am J Enol Vitic 47:206–216

Magliani W, Conti S, Gerloni M, Bertolotti D, Polonelli L (1997) Yeast killer systems. Clin Microbiol Rev 10:369–400

Magyar I, Panyik I (1989) Biological deacidification of wine with *Schizosaccharomyces pombe* entrapped in Ca-alginate gel. Am J Enol Vitic 40:233–240

Mannazzu I, Clementi F, Ciani M (2002) Strategies and criteria for the isolation and selection of autochthonous starters. In: Ciani M (ed) Biodiversity and biotechnology of wine yeasts. Research Signpost, Trivandrum, India, pp 19–33

Mendoza LM, Manca de Nadra MC, Farıas ME (2007) Kinetics and metabolic behaviour of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. Biotechnol Lett 29:1057–1063

Mora J, Barbas JI, Mulet A (1990) Growth of yeast species during the fermentation of musts inoculated with *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. Am J Enol Vitic 41:156–159

Moreira N, Mendes F, Guedes de Pinho P, Hogg T, Vasconcelos I (2008) Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as a pure and mixed cultures in grape must. Int J Food Microbiol 124:231–238

Musmanno RA, Di Maggio T, Coratza G (1999) Studies on strong and weak killer phenotypes of wine yeasts: production, activity of toxin in must, and its effect in mixed culture fermentation. J Appl Microbiol 87:932–938

Petering JE, Symons MR, Langridge P, Henschke PA (1991) Determination of killer yeast activity in fermenting grape juice by using a marked *Saccharomyces* wine yeast strain. Appl Environ Microbiol 57:3232–3236

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16:675–729

Ramon-Portugal F, Delia ML, Strehaiano P, Riba JP (1998) Mixed culture of killer and sensitive *Saccharomyces cerevisiae* strains in batch and continuous fermentations. World J Microbiol Biotechnol 14:83–87

Reed G, Nagodawithana TW (1988) Technology of yeast usage in wine making. Am J Enol Vitic 39:83–90

Ribereau-Gayon P, Peynaud E (1960) Traité d'Oenologie. Paris et Liege Librarie Polytechnique Ch. Béranger, Paris, pp 293–298

Rojas V, Gil JV, Pinaga F, Manzanares P (2001) Studies on acetate ester production by non-*Saccharomyces* wine yeasts. Int J Food Microbiol 70:283–289

Romano P, Suzzi G, Comi G, Zironi R, Maifreni M (1997) Glycerol and other fermentation products of apiculate wine yeasts. J Appl Microbiol 82:615–618

Rosini G, Ciani M (1988) Carattere killer ed ecologia dei *Saccharomyces cerevisiae* della vinificazione. Atti Accad Ital Vite Vino 40:311–318

Rosini G, Ciani M, Vaughan AE (1988) Vino Sagrantino D.O.C.: correlazione tra colture di *Saccharomyces cerevisiae* isolate dai vini e quelle presenti nei locali di vinificazione. Ann Microbiol 38:171–179

Sangorrín MP, Lopes CA, Giraudo MR, Caballero AC (2007) Diversity and killer behaviour of indigenous yeasts isolated from the fermentation vat surfaces in four Patagonian wineries. Int J Food Microbiol 119:351–357

Santos A, San MM, Bravo E, Marquina D (2009) PMKT2, a new killer toxin from *Pichia membranifaciens*, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. Microbiology 155:624–634

Shimizu K (1993) Killer yeasts. In: Fleet GH (ed) Wine Microbiology and Biotechnology. Harwood, Chur, pp 243–264

Snow PG, Gallender GF (1979) Deacidification of white table wines through partial fermentation by *Schizosaccharomyces pombe*. Am J Enol Vitic 30:45–48

Sponholz WR (1993) Wine spoilage by microorganisms. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood, Chur, pp 399–400

Ueda-Nishimura K, Mikata K (1999) A new yeast genus, *Tetrapisispora* gen. nov.: *Tetrapisispora iriomotensis* sp. nov., *Tetrapisispora nanseiensis* sp. nov. and *Tetrapisispora arboricola* sp. nov., from the Nansei Islands, and reclassification of *Kluyveromyces phaffii* (van der Walt) van der Walt as *Tetrapisispora phaffii* comb. nov. Int J Syst Bacteriol 49:1915–1924

Vagnoli P, Musmanno RA, Cresti S, Di Maggio T, Coratza G (1993) Occurrence of killer yeasts in spontaneous wine fermentations from the Tuscany Region of Italy. Appl Environ Microbiol 59:4037–4043

van Vuuren HJJ, Jacobs CJ (1992) Killer yeasts in the wine industry: a review. Am J Enol Vitic 43:119–128

Viana F, Gil JV, Vallés S, Manzanares P (2009) Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. Int J Food Microbiol 135:68–74

Yokotsuka K, Otaky A, Naitoh A, Tanaka H (1993) Controlled simultaneous deacidification and alcohol fermentation of high-acid grape must using two immobilized yeasts, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Am J Enol Vitic 44:371–377

Zironi R, Romano P, Suzzi G, Battistutta F, Comi G (1993) Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. Biotechnol Lett 15:235–238