Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

The zymocidial activity of *Tetrapisispora phaffii* in the control of *Hanseniaspora uvarum* during the early stages of winemaking

F. Comitini and M. Ciani

Dipartimento SAIFET, Sez. Microbiologia Alimentare, Industriale e Ambientale, Università Politecnica delle Marche, Via Brecce Bianche, Ancona, Italy

Keywords
Hanseniaspora uvarum, immobilization, killer toxin, *Tetrapisispora phaffii*, winemaking.

Correspondence
Maurizio Ciani, Dipartimento SAIFET sez. Microbiologia alimentare, industriale e ambientale, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy. E-mail: m.ciani@univpm.it

2009/1333: received 27 July 2009, revised and accepted 25 September 2009

doi:10.1111/j.1472-765X.2009.02754.x

Abstract

Aims: The yeast strain *Tetrapisispora phaffii* DBVPG 6706 (formerly *Kluyveromyces phaffii*) secretes a killer toxin (Kpkt) that has antimicrobial activity against apiculate yeasts. The aim of this study was to evaluate the killer activity of Kpkt towards *Hanseniaspora uvarum* under winemaking conditions.
Methods and Results: The zymocidial activity of Kpkt on *H. uvarum* was assayed in microfermentation trials inoculated with free and immobilized *T. phaffii* cells. The microbial evolution and fermentation profiles of the wines were evaluated to determine the effects of Kpkt on apiculate yeasts, in comparison with SO_2. The results indicate that the fungicidal activity of Kpkt against *H. uvarum* is stable for at least 14 days in wine, and the zymocin can control the proliferation of apiculate yeasts. The analytical composition of wines with the inoculum of *T. phaffii* immobilized cells did not differ from the wines with SO_2. In contrast to wines without this control of apiculate yeasts, an increase in ethyl acetate was seen.
Conclusions: *Tetrapisispora phaffii* is an excellent candidate for the biological control of undesired proliferation of apiculate yeasts during the first steps of fermentation.
Significance and Impact of the Study: *Tetrapisispora phaffii* cells in an immobilized form can be used as a biocontrol agent to reduce the need for SO_2 addition.

Introduction

The killer character of yeast was originally discovered in some strains of *Saccharomyces cerevisiae*, and it is associated with the secretion of proteins known as killer toxins that can kill sensitive target cells in a receptor-mediated process, without direct cell-to-cell contact (Bevan and Makower 1963; Bevan *et al.* 1973). The uniqueness of this phenomenon is related not only to the immunity of the killer yeasts to their own killer toxin but also to their susceptibility to the toxins that are secreted by other killer strains.

At present, killer yeasts have many potential applications in the biotechnology field, and the use of killer toxins offers a considerable advantage for these yeast strains under competitive conditions, against other sensitive microbial cells in their ecological environment (Philliskirk and Young 1975; Fleet 2003). This advantage has a scientific and commercial significance, because killer yeasts and their toxins have several applications in the industrial, agricultural and medicinal fields (Kitamoto *et al.* 1993; Cailliez *et al.* 1994; Walker *et al.* 1995; Lowes *et al.* 2000; Marquina *et al.* 2002; Schmitt and Breinig 2002). In food industry, killer yeasts and their toxins have many potential applications and have been proposed to combat contamination by wild yeasts (Palpacelli *et al.* 1991; Goretti *et al.* 2009; Liu and Tsao 2009).

An interesting application of this zymocidial activity in winemaking is seen in the killer yeasts that are used to control the proliferation of spoilage yeasts during the prefermentation phase. Wine fermentation is carried out

using nonsterile must in which there are generally wild yeasts that can affect the process, causing negative modifications to the final aroma of the wine (Lowes *et al.* 2000; Yap *et al.* 2000). Killer yeasts belonging to *S. cerevisiae* are currently used to initiate wine fermentation in winemaking and to improve the fermentation process and the quality of the final wine (van Vuuren and Jacobs 1992). However, the main limitations in the use of *S. cerevisiae* killer strains as selected starter strains is their narrow antiyeast spectrum: as it is restricted to sensitive *Saccharomyces* strains, it does not affect wild yeasts, such as those belonging to the genera *Hanseniaspora/Kloeckera, Pichia, Brettanomyces, Zygosaccharomyces* and *Saccharomycodes*, which represent the main targets of antimicrobial agent used in winemaking (van Vuuren and Jacobs 1992; Shimizu 1993). On the other hand, the total suppression of indigenous non-*Saccharomyces* species can reduce the aroma complexity of the final wines. However, natural multistarter cultures remain an uncontrolled process that can positively or negatively affect the final product.

The wild microflora is controlled using sulfur dioxide (SO_2), a chemical antimicrobial agent that is largely used in the food industries and that has selective antiseptic activities (Constantì *et al.* 1998). The addition of this antiseptic agent produces several risks; for instance, adverse effects on the respiratory system of humans and animals and damage to vegetation. For these reasons, the World Health Organization and the European Economic Community have highlighted the need to reduce the use of SO_2 as an antimicrobial agent in food products.

Following these indications and with the support of new winemaking technologies, during the last decades, winemakers significantly reduced the amounts of SO_2 added during the various stages of winemaking. On the other hand, some environmental and technological factors such as climatic condition, fungicide treatments, the degree of maturation of grapes, time of maceration or clarification of must led the presence and the development of wild yeast population particularly apiculate yeasts (Longo *et al.* 1991; Fleet *et al.* 2002). In this context, the use of a killer toxin to control the proliferation of apiculate yeasts at a prefermentative stage could further reduce the need for the use of chemical antiseptic agents.

Previous studies have shown that the yeast *Tetrapisispora phaffii* produces a killer toxin (i.e. KpKt) that is active on *Hansenispora/Kloeckera* wine yeasts under winemaking conditions, yeasts that are largely present at the prefermentative stages (Ciani and Fatichenti 2001; Comitini *et al.* 2004).

In view of the possible use of *T. phaffii* in the wine and beverage industries, the present study evaluates the zymocidial activity of *T. phaffii* under winemaking conditions.

Materials and methods

Micro-organisms and media

The following strains were used, which belong to the Industrial Yeast Collection of the University of Perugia (DBVPG): DBVPG 6076, the *T. phaffii* killer strain and DBVPG 3037, a Kpkt-sensitive strain of *Hanseniaspora uvarum*. The *S. cerevisiae* commercial strain called Uvaferm BC® (Lallemand Inc., France) was used as a yeast that is not sensitive to Kpkt.

The yeast strains were subcultured on malt agar at 6-month intervals and maintained at 6°C. The media used were malt agar (Difco), YPD (1% Bacto yeast extract, 1% Bacto peptone, 2% glucose), and WL Nutrient Agar (Oxoid, Hampshire, UK) for differentiation of wild yeasts from must samples (Pallmann *et al.* 2001).

Microfermentation trials were carried out using Trebbiano toscano grape must (pH 3·3; 168 g l^{-1} sugar; total acidity 7·3 g l^{-1}; 40·3 mg l^{-1} ammonia; 222·7 mg l^{-1} primary amino acids and SO_2 free). The must was pasteurized at 75°C for 15 min, before use.

Cell immobilization

Tetrapisispora phaffii cells for immobilization were grown in YPD at 25°C in a rotary shaker (150 rev min^{-1}, for 72 h). They were then harvested by centrifugation, washed three times in sterile distilled water and added to 100 ml of 2·5% sodium alginate (Carlo Erba, Milan, Italy). To induce gel formation, this solution was added dropwise to an aqueous 0·1 mol l^{-1} calcium chloride solution, using a peristaltic pump and maintaining the cell suspension under constant agitation at 25°C. After 1 h, the beads that were formed were washed several times with sterile distilled water and then used immediately.

The concentration of the immobilized cells was measured by melting 1 g of beads in 99 ml of 1% sodium citrate (w/v); then serial dilutions were made, and viable cell counts were carried out.

For the trials, 50 g of these spherical beads were inoculated into 250 ml sterile grape juice, to a final concentration of 20% beads for each condition (4×10^8 cells ml^{-1}).

Microfermentation trials

Five trials were prepared using the following modalities for the control of the proliferation of the apiculate yeasts:

i positive control: without *T. phaffii* cell inoculum and without SO_2 addition;

ii negative control: addition of 60 mg l^{-1} SO_2;

iii inoculum of *T. phaffi* as 10^6 free cells ml^{-1};
iv inoculum of *T. phaffii* as 10^7 free cells ml^{-1};
v inoculum of *T. phaffii* as 4×10^8 immobilized cells ml^{-1}.

In each trial, a suspension of *H. uvarum* DBVPG 3037 cells from an 18-h preculture was inoculated into 250 ml sterile grape juice, to achieve a final concentration of 10^5 cells ml^{-1}. After two days, an inoculum of the *S. cerevisiae* starter strain in the active dry yeast form was carried out, to obtain an initial concentration of 10^6 cells ml^{-1}. In the trial with the immobilized killer strain, the beads were added at a percentage ratio of 20% (w/v), with a final concentration of 4×10^8 cells ml^{-1}; and these were put into a bottle with a holed septum and submerged in the must.

The effect on *H. uvarum* DBVPG 3037 of the zymocidial activity of Kpkt from the free and immobilized *T. phaffii* cells was monitored by viable plate counting on WL nutrient agar. The toxin stability during the alcoholic fermentation was evaluated by means of a well-test assay following the indications of Ciani and Fatichenti (2001). To better estimate the Kpkt activity, the samples from each of the trials were concentrated before the well-test assay. For this, 10-ml samples were harvested, centrifuged and microfiltered (0·45 μm pore-size), and then they were concentrated 10-fold by means of an ultrafiltration step using 15-ml ultracentrifuge filter devices with a cut-off of 10 kDa (Comitini *et al.* 2004). The trials were carried out in duplicate.

Analytical determinations

Ethanol and volatile acidity (quantified by steam distillation) were measured following the Official EU Methods (Anonymous EC 2000). Acetaldehyde, ethyl acetate, acetoin and the higher alcohols were determined by GLC, using the procedure described by (Ciani *et al.* 2006).

Ammonia was assayed by diagnostic kits (no. 171-UV, Sigma Aldrich, St Louis, MI, USA), while primary amino acids (assimilable nitrogen) were evaluated following the procedures described by Dukes and Butzke (1998).

Statistical analysis

An analysis of variance (ANOVA) was applied to the experimental data. The values of means were analysed using the software Super ANOVA ver. 1.1 for Mac OS 9.1. The significant differences were determined by Duncan tests, and the results were considered significant if the associated *P*-value was below 0·01.

Results

The evolution of the viable cells in the microfermentations is shown in Fig. 1. Without any controlling agents, the apiculate strain, *H. uvarum*, increased by three logarithmic orders, from 10^5 to 10^8 CFU ml^{-1}, showing the maximum cell counts on the third day (Fig. 1a). After inoculation of the *S. cerevisiae* starter strain (day 2), the *H. uvarum* cell concentration decreased until these cells disappeared after day 10 (Fig. 1a). As expected, in the presence of sulfur dioxide from day 0, there was a more rapid death of the *H. uvarum* apiculate strain (disappeared after day 3) (Fig. 1b).

The biomass evolution of *H. uvarum* in the presence of the *T. phaffii* free killer cells from day 0 is shown in Fig. 1c,d. Here, *H. uvarum* did not reach the cell concentration of the positive control (10^8 CFU ml^{-1}), achieving cell counts after 3 days of *c.* 10^7 CFU ml^{-1} with both *T. phaffii* additions. Under these conditions, *T. phaffii* showed only partial control of the proliferation of the *H. uvarum* apiculate yeast population. Indeed, here *H. uvarum* persisted during the fermentation until day 10, as for the positive control (Fig. 1a). The biomass evolution of *H. uvarum* together with the immobilized *T. phaffii* cells (Fig. 1e) showed similar behaviour to the trial carried out with SO_2 (Fig. 1b): in the presence of these beads of *T. phaffi*, the numbers of the *H. uvarum* strain gradually decreased, with 10^3 CFU ml^{-1} after three days (days 3/4), and then disappeared completely after day 8. The biomass evolution of *S. cerevisiae* was very similar across all of these trials (Fig. 1), indicating that there was no significant effect of the inoculation of *T. phaffii* on the inoculated *S. cerevisiae* starter strain.

The results of the well-test assay indicate that both free and immobilized *T. phaffii* cells showed zymocidial activity during all of the fermentation process, persisting in the must-wine for the full 14-day sampling time (Table 1). The presence and the level of zymocidial activity appears not to be related to the killer strain inoculation level (not significantly different for the addition of 10^6 or 10^7 free cells ml^{-1}). On the other hand, the immobilization procedure (with 4×10^8 immobilized cells ml^{-1}) appeared to significantly increase the amounts of zymocin released, because a linear relationship was seen between the logarithm of killer toxin concentration and the diameter of inhibition halo (Ciani and Fatichenti 2001).

The main enological fermentation parameters analysed showed some differences across these different trials at the end fermentation (at 14 day) (Table 2). In particular, as expected, the ethanol formed was slightly reduced in the presence of the non-*Saccharomyces* species. The volatile acidity was significantly higher only in the positive control trial, where there were high levels of the *H. uvarum* apiculate yeasts, which persisted for a long time.

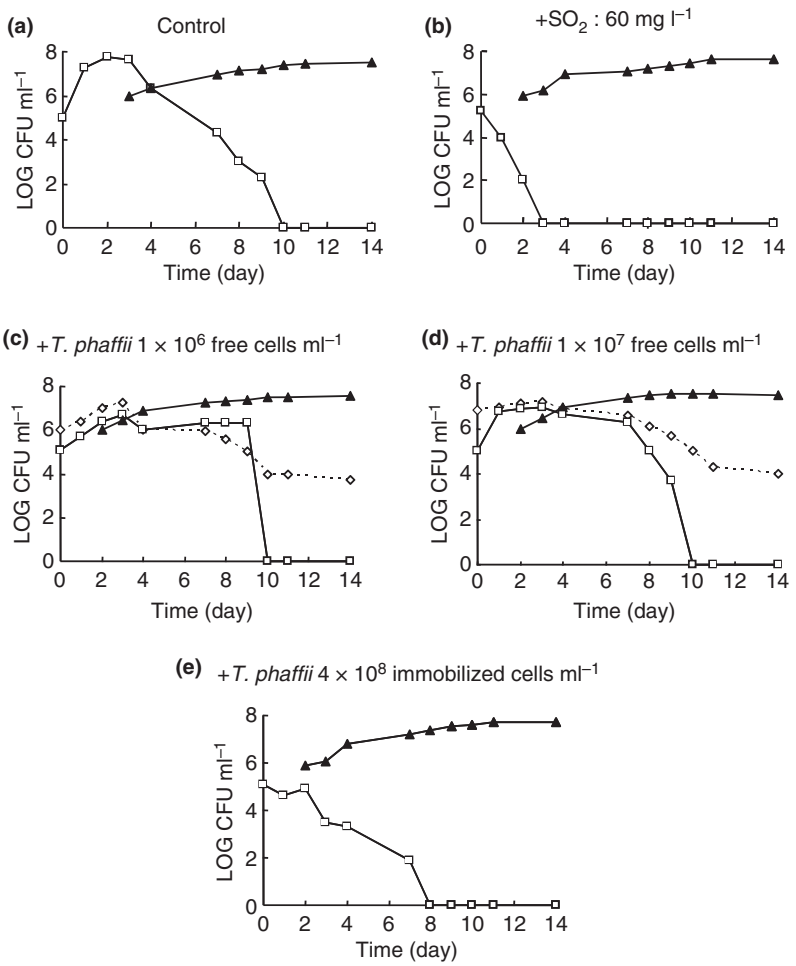

Figure 1 Evolution of yeast inoculated populations during microfermentations. As indicated: (a) positive control, without *Tetrapisispora phaffii* cells and without SO_2 addition; (b) negative control, with the addition of 60 mg l^{-1} of SO_2; (c) inoculum of *T. phaffii* at 10^6 free cells ml^{-1}; (d) inoculum of *T. phaffii* at 10^7 free cells ml^{-1}; (e) inoculum of immobilized *T. phaffii* at 4×10^8 cells ml^{-1}. Yeast populations: ▲, pure *Saccharomyces cerevisiae*; □, *Hanseniaspora uvarum* sensitive strain; ◇, free *T. phaffii* cells. The data are means of two independent experiments.

Table 1 Inhibition halos from samples collected during the fermentation trials inoculated as indicated. The zymocin was tested against the *Hanseniaspora uvarum* DBVPG 3037 sensitive strain in a well-test assay

	Diameter of inhibition halos (mm)		
Sampling time (d)	Free *Tetrapisispora phaffii* (10^6 cells ml^{-1})	Free *T. phaffii* (10^7 cells ml^{-1})	Immobilized *T. phaffii* cells (4×10^8 cells ml^{-1})
1·0	9·2 ± 0·5	9·1 ± 0·2	12·2 ± 0·8
1·5	11·0 ± 0·7	11·0 ± 0·2	13·1 ± 0·5
2·0	9·6 ± 0·3	10·1 ± 0·5	15·3 ± 0·6
2·5	10·1 ± 0·1	10·3 ± 0·9	15·0 ± 0·5
3·0	10·0 ± 0·1	10·2 ± 0·5	15·5 ± 0·7
7·0	10·2 ± 0·5	10·5 ± 0·4	15·1 ± 0·7
10	10·4 ± 0·7	10·8 ± 0·1	15·3 ± 0·2
14	9·5 ± 0·7	10·3 ± 0·2	14·1 ± 0·1

The data are means ± SD.

The results of the analysis of the main fermentation by-products at the end of the trials also show differences across these trials (Fig. 2). The positive control trial was characterized by a low concentrations of acetaldehyde and high concentrations of ethyl acetate production, which will have been caused by the uncontrolled growth of *H. uvarum*. As would be expected, the SO_2 addition reduced the ethyl acetate production and slightly increased the concentration of acetaldehyde (not significantly different). With the addition of the free *T. phaffii* cells, this confirmed the results of the *H. uvarum* biomass evolution where limited control of *H. uvarum* proliferation was seen, with a consistently high production of ethyl acetate, a trend that was comparable with that shown by the positive control. In these free *T. phaffii* cell addition trials, an increase in acetaldehyde production was also seen. The use of immobilized *T. phaffii* cells significantly reduced the ethyl acetate production (in comparison with the positive control) to a concentration similar to that with SO_2 addition, while the concentration of acetaldehyde increased. For the higher alcohols, there were no significant differences across the trials.

Table 2 Main enological characters evaluated at the end of the microfermentations (day 14)

Fermentation conditions	pH	Total acidity (g l^{-1})	Volatile acidity (g l^{-1})	Ethanol (% vol.)
Positive control: without *Tetrapisispora phaffii* cell inoculum and without SO_2 addition	3·25	7·95 ± 1·06	0·88 ± 0·13	8·6 ± 0·1
Negative control: addition of 60 mg l^{-1} SO_2	3·18	5·65 ± 0·21	0·38 ± 0·01	9·2 ± 0·1
Free *T. phaffii* cells (10^6 cells ml^{-1})	3·24	6·75 ± 0·07	0·42 ± 0·02	8·2 ± 0·2
Free *T. phaffi* cells (10^7 cells ml^{-1})	3·24	6·40 ± 0·14	0·34 ± 0·00	8·3 ± 0·2
Immobilized *T. phaffii* beads (4×10^8 cells ml^{-1})	3·27	5·80 ± 0·84	0·32 ± 0·03	8·4 ± 0·1

The data are means ± SD.

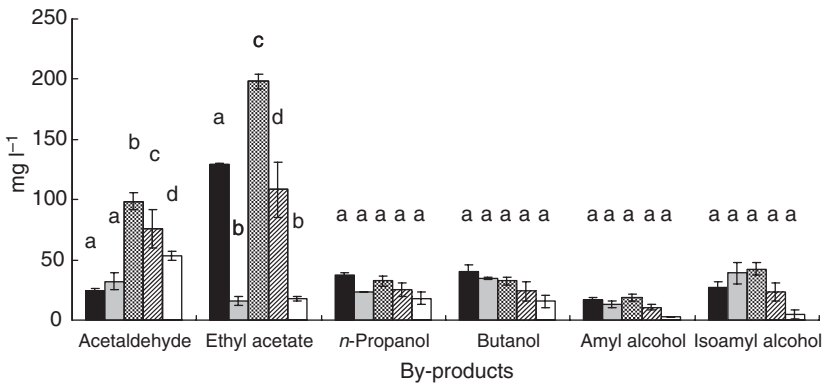

Figure 2 Fermentation by-products evaluated at the end of the trials. (■) positive control, without *Tetrapisispora phaffii* cells and without SO_2 addition; (▩) negative control, with addition of 60 mg l^{-1} SO_2; (▩) inoculum of *T. phaffii* at 10^6 free cells ml^{-1}; (▨) inoculum of *T. phaffii* at 10^7 free cells ml^{-1}; (□) inoculum of immobilized *T. phaffii* at 4×10^8 cells ml^{-1}. The data are means ± SD. Values displaying different letters within each column are different according to the Duncan test (0·01%).

Discussion

In the present study, we proposed the use of a killer yeast, *T. phaffii*, that is characterized by a wide spectrum of zymocidial activity, to control wild yeast belonging to the *H. uvarum*-sensitive yeasts. The occurrence of the killer strains has also been examined in other substrates, with high sugar contents, such as sugarcane (Morais *et al.* 1997; Soares and Sato 1999) and during fermentative processes carried out in nonsterile media, like grape must.

In winemaking, the widespread occurrence of killer wine yeasts has been demonstrated in *S. cerevisiae* strains isolated from wineries in different regions of the world (Shimizu 1993). This extensive incidence has encouraged interest in the enological significance of killer wine yeasts. However, the main limitations in the use of *S. cerevisiae* killer strains as selected starters is in their narrow anti-yeast spectrum: as it is restricted to sensitive *Saccharomyces* strains, it does not affect the wild yeast flora that represent the main targets of antimicrobial agents in winemaking.

Indeed, during fermentation, these wild micro-organisms could produce a wide variety of antagonistic primary and secondary metabolites including ethanol, weak organic acids, middle-chain fatty acids, acetaldehyde, diacetyl, CO_2 and even antimicrobial compounds, such as killer toxins and bacteriocins. In the control of the proliferation of undesired wild yeasts, the use of killer yeasts could be an alternative to chemical antiseptic agents, such as SO_2.

Our results show that the inoculation of *T. phaffii* into natural grape juice during microvinifications can indeed act on the *H. uvarum* strain. The results of well-test assay, the *H. uvarum* biomass evolution, and the analytical compositions of the final wines together indicate a reduction in the presence and limitation of the metabolic activity of this *H. uvarum* apiculate strain. However, using free cells of *T. phaffii*, there was no significant control of *H. uvarum* apiculate yeasts, which was accompanied by the slight increase in the concentrations of ethyl acetate (only in 10^6 free cells ml^{-1} trial) and acetaldehyde. In contrast, with the immobilized *T. phaffii* cells, *H. uvarum* showed a weak development, which also maintained a low concentration of ethyl acetate. The better control of sensitive yeast by immobilized *T. phaffii* cells was probably because of the higher cell concentration in the beads (in resting cells condition) in comparison with that of free cells trials. The control of

ethyl acetate is of particular interest, as its levels in wine mainly depend on the contributions from such apiculate yeasts (Ciani *et al.* 2006). At low concentrations, ethyl acetate can contribute to the fruity notes of a wine and add to the general complexity (Gil *et al.* 1996); however, when a threshold taste level (of 150 mg l^{-1}; Jackson 1994) is surpassed, the ethyl acetate causes a sour-vinegar off odour. In this context, modulated use of immobilized *T. phaffii* cells should be useful to obtain the desired levels of ethyl acetate production and also for the production of the other esters.

Acknowledgement

This work was partially financially supported by Lallemand Inc. Succursale Italiana, Verona Italy.

References

Anonymous EC (2000) Official Journal of the European Community. Community reference methods for the analysis of spirit drinks. Reg. EC no. 2870/00. 19 December 2000. Brussels.

Bevan, E.A. and Makower, M. (1963) The physiological basis of the killer character in yeast. *Proc XIth Int Congr Genet* **1**, 202–203.

Bevan, E.A., Herring, A.J. and Mitchell, D.J. (1973) Preliminary characterization of two species of dsRNA in yeast and their relationship to the ''killer'' character. *Nature* **245**, 81–86.

Cailliez, J.C., Seguy, N., Aliouat, E.M., Polonelli, L., Camus, D. and Dei-Cas, E. (1994) The yeast killer phenomenon: a hypothetical way to control *Pneumocystis carinii pneumonia*. *Med Hypo* **43**, 167–171.

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

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

Constantì, M., Reguant, C., Poblet, M., Zamora, F., Mas, A. and Guillamon, J.M. (1998) Molecular analysis of yeast population dynamics: Effect of sulphur dioxide and inoculum on must fermentation. *Int J Food Microbiol* **41**, 169–175.

Dukes, B.C. and Butzke, C.E. (1998) Rapid determination of primary amino acids in grape juice using an *o*-Phthaldialdehyde/*N*-Acetyl-l-Cysteine Spectrophotometric Assay. *Am J Enol Vitic* **49**, 125–134.

Fleet, G.H. (2003) Yeast interactions and wine flavour. *Int J Food Microbiol* **86**, 11–22.

Fleet, G.H., Prakitchaiwattana, C., Beh, A.L. and Heard, G. (2002) The yeast ecology of wine grapes. In *Biodiversity and biotechnology of wine yeast* ed. Ciani, M. pp. 1–17 Kerala, India: Research Signpost.

Gil, J.V., Mateo, J.J., Jiménez, M., Pastor, A. and Huerta, T. (1996) Aroma compounds in wine influenced by apiculate yeasts. *J Food Sci* **61**, 1247–1266.

Goretti, M., Turchetti, B., Buratta, M., Branda, E., Corazzi, L., Vaughan-Martini, A. and Buzzini, P. (2009) *In vitro* antimycotic activity of a *Williopsis saturnus* killer protein against food spoilage yeasts. *Int J Food Microbiol* **131**, 178–182.

Jackson, R. (1994) *Wine Science. Principles and Applications*. San Diego, USA: Academic Press, pp. 178–219.

Kitamoto, H.K., Ohmomo, S. and Nakahara, T. (1993) Selection of killer yeasts (*Kluyveromyces lactis*) to prevent aerobic deterioration in silage making. *J Dairy Sci* **76**, 803–811.

Liu, S.Q. and Tsao, M. (2009) Inhibition of spoilage yeasts in cheese by killer yeast *Williopsis saturnus* var. *saturnus*. *Int J Food Microbiol* **131**, 280–282.

Longo, E., Cansado, J., Agrelo, D. and Villa, T. (1991) Effect of climatic conditions on yeast diversity in grape must from North-west Spain. *Am J Enol Vitic* **42**, 141–144.

Lowes, K.F., Shearman, C.A., Payne, J., MacKenzie, D., Archer, D.B., Merry, R.J. and Gasson, M.J. (2000) Prevention of yeast spoilage in feed and food by the yeast mycocine HMK. *Appl Environ Microbiol* **66**, 1066–1076.

Marquina, D., Santos, A. and Peinado, J.M. (2002) Biology of killer yeasts. *Int Microbiol* **5**, 65–71.

Morais, P.B., Rosa, C.A., Linardi, V.R., Pataro, C. and Maia, A.B.R.A. (1997) Characterization and succession of yeast populations associated with spontaneous fermentations during the production of Brazilian sugarcane aguardente. *World J Microbiol Biotechnol* **13**, 241–243.

Pallmann, C.L., Brown, J., Olineka, T.L., Cocolin, L., Mills, D.A. and Bisson, L.F. (2001) Use of WL medium to profile native flora fermentations. *Am J Enol Vitic* **52**, 198–203.

Palpacelli, V., Ciani, M. and Rosini, G. (1991) Activity of a different 'killer' yeast on strains of yeast species undesirable in the food industry. *FEMS Microbiol Lett* **84**, 75–78.

Phillipskirk, G. and Young, T.W. (1975) The occurrence of killer character in yeasts of various genera. *Antonie Van Leeuwenhoek* **41**, 147–151.

Schmitt, M.J. and Breinig, F. (2002) The viral killer system in yeast: from molecular biology to application. *FEMS Microbiol Rev* **26**, 257–276.

Shimizu, K. (1993) Killer Yeasts. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 243–264 Chur, Switzerland: Harwood Academic.

Soares, G.A.M. and Sato, H.H. (1999) Killer toxin of *Saccharomyces cerevisiae* Y500-4L active against Fleischmann and Itaiquara commercial brands of yeast. *Rev Microbiol* **30**, 253–257.

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

Walker, G.M., McLeod, A.H. and Hodgson, V.J. (1995) Interactions between killer yeasts and pathogenic fungi. *FEMS Microbiol Lett* **127**, 213–222.

Yap, N.A., de Barros Lopes, M., Langridge, P. and Henschke, P.A. (2000) The incidence of killer activity of non-*Saccharomyces* yeasts towards indigenous yeast species of grape must: potential application in wine fermentation. *J Appl Microbiol* **89**, 381–389.

