Biotechnol Lett (2007) 29:1057–1063
DOI 10.1007/s10529-007-9355-0

ORIGINAL RESEARCH PAPER

Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains

Lucía M. Mendoza · María C. Manca de Nadra · Marta E. Farías

Received: 22 January 2007 / Accepted: 22 February 2007 / Published online: 18 April 2007
© Springer Science+Business Media B.V. 2007

Abstract The kinetics and metabolic behavior of *Kloeckera apiculata* mc1 and *Saccharomyces cerevisiae* mc2 in composite culture was investigated. *K. apiculata* showed a higher viability through the fermentation; however the maximum cell density of both yeasts decreased. This behavior was not due to ethanol concentration, killer toxins production or competition for assimilable nitrogenous compounds between both yeasts. Despite the consistent production of secondary products by single culture of *K. apiculata*, an increase of these compounds was not observed in mixed culture. These results contribute to a better understanding of the behavior of non-*Saccharomyces* yeasts and their potential application in the wine industry.

Keywords Mixed culture · Non-*Saccharomyces* yeast · Wine

L. M. Mendoza · M. C. M. de Nadra · M. E. Farías
Centro de Referencia para Lactobacillus (CERELA), Chacabuco 145, 4000 Tucuman, Argentina

M. C. M. de Nadra · M. E. Farías (✉)
Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 491, 4000 Tucuman, Argentina
e-mail: mfarias@cerela.org.ar

Introduction

In traditional winemaking, natural (spontaneous) fermentation of grape juice is carried out by a sequence of different yeast species. The early stages are dominated by the growth of non-*Saccharomyces* yeasts, characterized by a low fermentative power (Fleet and Heard 1993). The apiculate yeasts belonging to genera *Kloeckera/Hanseniaspora* or other genera such as *Candida*, *Pichia* and *Metschnikowia* initiate the fermentation (Fleet 2003; Povhe Jemec et al. 2001; Fleet and Heard 1993). After 3–4 days these yeast die off, and are replaced by the strongly fermentative yeast (*Saccharomyces cerevisiae*) that continue and finish the fermentation process (Amerine et al. 1980; Martini 1993).

Some studies, however, showed that non-*Saccharomyces* yeast survive during the natural and inoculated fermentations of grape juice for longer periods than previously thought. In addition to ethanol and carbon dioxide, during the fermentation these yeast release secondary products such as higher alcohols, esters, acids, carbonyl compounds important to the sensory characteristics of wines (Romano et al. 1997, 2003; Ciani and Maccarelli 1998; Egli et al. 1998). Therefore, the practical benefit of the physiological and metabolic properties of the non-*Saccharomyces* yeast could be important in winemaking (Ciani 2001; Zohre and Erten 2002). In this context, interactive behaviors between the different species need to be considered.

In the present study, we report on the fermentation behavior and the metabolic interactions of mixed cultures of *Saccharomyces cerevisiae* mc2 strain and a potential non-*Saccharomyces* wine yeast starter species, *Kloeckera apiculata* mc1.

Materials and methods

Microorganisms

Kloeckera apiculata mc1 (apiculate yeast) and *Saccharomyces cerevisiae* mc2 (elliptic yeast) were isolated from Argentinean wines.

Culture medium and growth conditions

The yeasts were inoculated in the basal medium containing: 10 g yeast extract; 1.0 ml between 80 and 170 ml grape juice per litre distilled water, pH 5.5. Medium was treated at 90°C for 10 min and was inoculated with 10^6 cells ml^{-1} from 14 h pre-cultures grown in the same medium as follows: (i) individual pure culture of *K. apiculata* and *S. cerevisiae*; (ii) mixed culture of both yeast strains in the ratio 1:1. All cultures were incubated for 10 days at 30°C without agitation.

Determination of microbial growth and differential enumeration

Differential enumeration of wine yeasts in mixed culture was realized by plating on the following two media: (i) malt/agar medium (containing per litre: 20 g glucose, 1 g peptone, 20 g yeast extract, 3 g malt extract and 20 g agar, pH 6.8). The plates were incubated at 30°C for 3–4 days (Gil 1993). This medium allows non-*Saccharomyces* to be distinguished morphologically from *Saccharomyces* yeast colonies. *S. cerevisiae* formed colonies of brilliant white-cream with spherical and protuberant form with regular borders. The colonies of *K. apiculata* were opaque white-gray, smoothed or flattened form with lightly irregular borders and of tonality different to the rest of the colony. (ii) Modified malt agar medium by addition of 40 mg Bromophenol Blue l^{-1} with pH range: from 3 (yellow) to 4.6 (blue). The medium was adjusted at pH 4.6 and the plates were incubated at 30°C for 2–3 days. Non-*Saccharomyces* yeast colonies were blue while *Saccharomyces* formed yellow colonies. This behavior is related to differences in the metabolism of wine yeasts.

Fitting of yeasts death curves

The death curve of *K. apiculata* in pure culture was fitted using Eq. 1

$$\mathrm{Log}\ (N_t/N_0) = \mathrm{c} + \exp(a + bt)$$

The death curves of pure culture of *S. cerevisiae* and both yeasts in mixed cultures were fitted using Eq. 2

$$\mathrm{Log}\ (N_t/N_0) = a + bt$$

where N_0 (c.f.u ml^{-1}) is the cell concentration at the onset of death, N_t (c.f.u ml^{-1}) is the cell concentration at time t (days) and b (d^{-1}) is the death rate. The fittings were done with Statistica for Windows Release 5.0 using the least-squares algorithm of Levenberg-Marquadt (Truong-Meyer et al.1997).

Killer activity

For killer activity screening reference strains were used: *S. cerevisae* YAT 679 (K1 type), *S. cerevisae* NCYC 738 (K2 type), C. glabrata NCYC 388 (K4 type), *Kluyveromyces drosophilarum* NCYC 575 (K10 type) and a killer sensitive strain of *S. cerevisiae* P351 (PROIMI yeast collection). The tests were performed in plates of MB medium (containing per litre: 10 g yeast extract, 20 g peptone, 20 g glucose, 20 g agar, 0.03 g methylene blue, 0.1 M citrate/phosphate buffer) with different pH values (pH 3.5, 4.5 and 5). Strains are scored as killers when the inoculated strain is surrounded by a clear zone in which no growth of the seeded sensitive strain occurs, bounded by a zone of dead cells which stain dark blue in the presence of methylene blue. The sensitive character was detected by the formation of clear halos when a killer strain was seeded on the lawn of the strains to be tested. The strain that did not respond to both reference strains was considered as neutral. The plates were incubated 3–5 days at 18°C and 2 days at 28°C. In general, the temperature of incubation is 18°C because killer toxins are rapidly inactived at temperature in excess of 20°C. However, toxin

material tends to be more thermostable in agar and may be incubated at 28–30°C for 1–2 days (Young and Yagiu 1978).

Analytical determinations

Determination of the concentrations of sugars and fermentation products

Cell-free samples were obtained by centrifugation of the growth medium. Samples were stored at −20°C until analysis. The ethanol, glucose, fructose, acetic acid and glycerol concentrations were determined by HPLC (ISCO 2350, Software Peak Simple II) using a column HPX.87H (Bio-Rad, 300 × 7.8 mm). The column was eluted at 45°C with a degassed mobile phase containing 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. All the compounds were determined with a RI detector (Knauer).

Ethyl acetate was determined by gas chromatography using a HP5 column (30 m × 0.32 µm; film thickness 0.25 µm). Both injector and detector were operated at 220°C. The carrier gases were H_2 at 35 ml min^{-1} and N_2 at 36 ml min^{-1}. The column was programmed at 40°C for 2 min, rising to 160°C at a rate of 3°C min^{-1}, and 30 min at 160°C.

Samples were analyzed in triplicate. The identification and quantification of compounds were carried out by comparing retention time and concentration with standard solution (Sigma).

Utilization of nitrogenous compounds by mixed culture of wine yeasts

Proteins were determined in the supernatant by the method of Bradford with bovine serum albumin as standard.

At different incubation times, cells were centrifuged and supernatants were assayed for proteolytic activity. The modified Cd-ninhydrin method, with grape juice autoclaved as substrate, was utilized. After 1 h incubation at 30°C, the reaction was stopped by the addition of 0.65 ml 24% w/v trichloroacetic acid (TCA). A control precipitated with TCA immediately before incubation was conducted in all cases. To a sample of TCA supernant, 1.7 ml Cd-ninhydrin reagent was added and the mix was heated for 5 min at 84°C. After cooling, the absorbance was read at 507 nm. The absorbance for each sample was adjusted to the value produced by 1 mmol l^{-1} leucine solution.

Total free amino acids were quantified in supernatants using the cadmium ninhydrin reagent by the method of Doi et al (1981), with L-leucine as standard.

Ammonia was determined by the method proposed by Russel (1944).

Statistical analysis

An analysis of variance (ANOVA) was applied to the experimental data. The values means were analyzed using the software statistical MINITAB for Windows, version 14. The significant differences were determined by Tukey tests and the results were considered significant if the associated *P* value was below 0.05.

Results and discussion

Growth and survival of *K. apiculata* and *S. cerevisiae* in pure and mixed cultures

Figure 1 shows the evolution of yeast population during fermentation in the basal medium. *K. apiculata* in pure culture reached a maximum of 3×10^7 c.f.u ml^{-1} after the 3 days at 30°C and then declined. However, pure culture of *S. cerevisiae* grew for longer, reached its highest cell concentration (1.4×10^8 c.f.u ml^{-1}) after 6 days of fermentation. In mixed culture both wine yeasts showed a cell

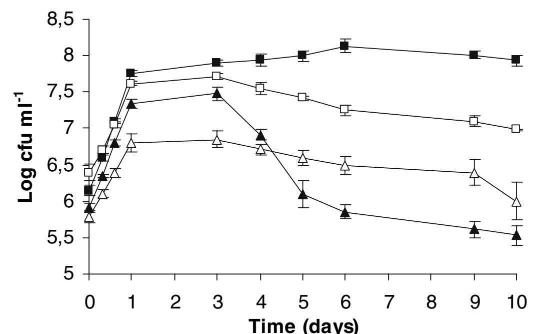

Fig. 1 Yeast population evolution of pure *K. apiculata* culture (▲), pure *S. cerevisiae* culture (■), mixed *K. apiculata* culture (△) and mixed *S. cerevisiae* culture (□) in grape juice medium. Values for colony forming units are the means of results obtained from three fermentations. Vertical bars represent standard deviation

concentration lower than in pure culture, the maximal population decreased to 5.9×10^6 c.f.u ml^{-1} and 5.5×10^7 c.f.u ml^{-1} for *K. apiculata* and *S. cerevisiae*, respectively.

Both yeasts grew at lower rates (values estimated from plots of the log of c.f.u ml^{-1} against time, using the straight line of the exponential growth phase) than in its respective pure cultures (30 and 23% for *K. apiculata* and *S. cerevisiae*, respectively). These results are in accordance with results reported by other authors. Moreira et al. (2005) determined a specific growth rate of 0.38 h^{-1} for pure cultures of *S. cerevisiae* and *Hanseniaspora uvarum*; however, this value decreased when these yeast were grown in mixed culture (0.33 and 0.26 h^{-1} for *S. cerevisiae* and *H. uvarum*, respectively). However, the apiculate cells remained viable during longer period than in pure culture and the elliptic yeast started to lose viability after 3 days of fermentation. Table 1 shows that after 10 days of incubation of *K apiculata* in co-culture condition, an increase of 4% of the relative growth of was observed with linear death kinetic (death rate = 0.12 d^{-1}), while in pure culture this yeast showed a 6.42% loss of viability with exponential death kinetic (death rate = 0.51 d^{-1}). The different types of death kinetics of *K. apiculata* depending on whether the yeast grows as single or mixed culture, reflects that the causes of the apiculate yeast death were not the same in both cultures. Our results are in agreement with that observed by Nissen and Arneborg (2003), who find that the death kinetics of *Kl. thermotolerans* and *T. delbruekii* in mixed culture have not been the same.

The relative growth of *Saccharomyces cerevisiae* in pure culture increased by 29%, but only by 9% in co-culture. Pure and mixed cultures of the elliptic yeast showed linear kinetic of death, being the death rate 0.09 and 0.15 d^{-1}, respectively.

Metabolic characteristics of wine yeasts in single and composite cultures

The main sugars present in the basal medium, glucose (18 ± 0.5 g l^{-1}) and fructose (15.4 ± 0.5 g l^{-1}) were fully consumed after 3 days of fermentation in pure and mixed cultures. The rate of glucose consumption was higher than fructose in different cultures but not show differences between the pure cultures of both yeasts (approximately 0.4 and 0.27 h^{-1} for glucose and fructose, respectively) (Table 2). However in mixed culture significant differences were found for both consumption rates (0.59 and 0.34 h^{-1} for glucose and fructose, respectively).

The highest ethanol concentration was determined in pure cultures of *S. cerevisiae*. When the elliptic yeast was co-inoculated with *K. apiculata*, even if the alcoholic fermentation was carried out with same rate than in pure culture of *Saccharomyces*, the ethanol concentration was lower. It is generally assumed that ethanol can reach concentrations leading to cell death of certain yeast species (Ludovico et al. 2001; Fleet 2003). Alcohol concentrations did not responsible of the lower biomass of both yeasts reached in co-inoculated cultures since in mixed trial a reduction of ethanol production related to the pure cultures was observed.

Table 1 Relative growth and death kinetics of *K. apiculata* and *S. cerevisiae* in pure and mixed cultures

Yeasts cultures	Relative growth (%)[a]			Death kinetics[b]	
	3	6	10	Curve shape	Rate (d^{-1})
K. apiculata					
Pure	26	− 0.96	− 6.42	Exponential	0.51
Mixed	18	12	4	Linear	0.12
S. cerevisiae					
Pure	29	37	29	Linear	0.09
Mixed	21	13	9	Linear	0.15

[a] Relative growth (%) = $(X_i - X_0/X_0) \times 100$. X_0: initial viable cell number; X_i: viable cell number after 3, 6 or 10 days. Negative values indicate cellular death

[b] Death kinetics were fitted using an exponential model Log (N_t/N_0) = c + exp($a + bt$) and a lineal model Log $(N_t/N_0) = a + bt$

Data are means of three fermentations

Table 2 Metabolic characteristics of wine yeasts in single and composite cultures

Cultures	Ethanol ($g\ l^{-1}$)	Volatile acidity ($g\ l^{-1}$)	Glycerol ($g\ l^{-1}$)	Ethyl acetate ($mg\ l^{-1}$)	Glucose consumption rate (h^{-1})	Fructose consumption rate (h^{-1})	Assimilable nitrogenous compounds used ($mg\ l^{-1}$)
K. apiculata	10.98 ± 0.20^{a}	0.98 ± 0.03^{a}	1.65 ± 0.04^{a}	220.5 ± 19.2^{a}	0.49 ± 0.02^{a}	0.25 ± 0.01^{a}	225 ± 10.5^{a}
S. cerevisiae	11.46 ± 0.23^{b}	0.54 ± 0.05^{b}	1.21 ± 0.04^{b}	55.4 ± 5.1^{b}	0.50 ± 0.02^{a}	0.26 ± 0.02^{a}	280 ± 7.8^{b}
K. apiculata + S. cerevisiae	9.67 ± 0.17^{a}	0.68 ± 0.04^{c}	1.47 ± 0.08^{c}	98 ± 19.2^{c}	0.59 ± 0.03^{b}	0.34 ± 0.01^{b}	255 ± 9.4^{c}

The amount of sugar at the start was 33.4 $g\ l^{-1}$

The initial yeast assimilable nitrogenous compounds were 545 $mg\ l^{-1}$ (325 $mg\ l^{-1}$ ammonia and 220 $mg\ l^{-1}$ primary amino acids)

Values are means ± standard deviations

Values displaying different superscript letters within each column are different according to the Tukey test

The high production of acetic acid is recognized as a common pattern in apiculate yeasts and for this characteristic; they have been considered for long time as spoilage yeasts (Romano et al 2003). Despite the consistent production of acetic acid in pure culture, *K. apiculata* did not cause an increase in volatile acidity in mixed cultures. Also the apiculate yeasts formed higher amounts of ethyl acetate in pure cultures. However in co-culture with the elliptic yeast, the level of ethyl acetate achieved could contribute to the fruity noted and add to the general complexity. These results are in accordance with results reported by other authors. Ciani et al. (2006), showed that in mixed or sequential cultures of *H. uvarum*/*S. cerevisiae*, volatile acidity is lower than that seen in pure cultures of *H. uvarum*.

Glycerol is a wine constituent related to yeast metabolism which contributes to the sweetness, viscosity and smoothness of wine (Gardner et al. 1993; Ciani and Ferraro 1996).

In the present study the production of glycerol was greater by non-*Saccharomyces* yeast and in mixed culture.

Pure cultures of *K. apiculata* presented lower consumption of the assimilable nitrogenous compounds than elliptic yeast. In co-culture conditions, utilization of these compounds (present in non-deficient concentrations: 545 $mg\ l^{-1}$) decreased with respect to *S. cerevisiae* in pure culture. Considering that in a mixed trial, less nitrogen was used than in pure culture (Table 2), the lower biomass of both yeasts in co-inoculation condition was not due to a competition for assimilable nitrogenous compounds.

Table 3 shows the evolution of extracellular protease production and protein concentration during the growth of pure and mixed cultures of the yeast strains in the basal media. In all experiences the maximal proteolytic activity were detected at 72 h yeasts growth. Single culture of *S. cerevisiae* showed the highest exoprotease activity. It was observed correlation between proteolytic activity of yeasts and the evolution of protein concentrations in the media. In composite culture the proteolytic activity and its effectiveness on the protein decreased in comparison with pure cultures of the elliptic yeast, however was higher respect to single culture of the apiculate yeast. It can infer that the higher viability of *K. apiculata* observed in composite culture (Table 1) could be explained by the high and more effective proteolytic

Table 3 Protein degradation by exoproteases of non-*Saccharomyces* and *Saccharomyces* yeasts

Yeasts strains	Proteolityc activity (mM)			Protein concentrations (mg l^{-1})[b]		
	3 days[a]	6 days	10 days	3 days	6 days	10 days
K. apiculata	0.17 ± 0.006[a]	0.04 ± 0.002[a]	0.06 ± 0.001[a]	73.55 ± 4.3[a]	73.45 ± 3.1[a]	72.30 ± 2.5[a]
S. cerevisiae	0.25 ± 0.008[b]	0.07 ± 0.004[b]	0.09 ± 0.002[b]	51.50 ± 3.1[b]	50.70 ± 2.7[b]	49.25 ± 3.3[b]
K. apiculata + *S. cerevisiae*	0.19 ± 0.007[c]	0.10 ± 0.003[c]	0.08 ± 0.002[c]	58.25 ± 2.8[c]	57.25 ± 3.6[c]	56.05 ± 2.7[c]

[a] Time of incubation at 28°C in the basal medium

[b] The initial content of proteins in the basal medium was 86 mg l^{-1}

Data are means of three fermentations ± standard deviation. Values not sharing the same superscript letter within each column are different according to the Tukey test

activity observed in this condition. The proteolytic system of *Saccharomyces cerevisiae* could be release some essential amino acids for the growth of the apiculate yeast.

Killer activity of wine yeasts

During wine fermentations, yeasts can produce, beside ethanol, other toxic compounds, namely killer toxins (Fleet 2003). To determine if these compounds, produced by *S. cerevisiae* and *K. apiculata*, were involved in the diminution of maximum cell population in mixed culture, killer activity of the both wine yeast strains used in the present work was tested against the reference killer toxins K1, K2, K4 and K10 (Table 4). Tests revealed that *S. cerevisiae* was killer-sensitive against the reference killer toxins (phenotype K^{-} R^{+}) and *K. apiculata* was killer-neutral (K^{-} R^{-}). Additionally, none of the strains were killer positive towards the killer sensitive strain (*S. cerevisiae* P351). The variation of pH from 3.6 to 5.0 gave similar results. Similar results were reported by Pérez-Nevado et al. (2006), who studying the cellular death of two non-*Saccharomyces* wine-related yeasts in mixed fermentations with *S. cerevisiae* find that the former strains were killer neutral, while *Saccharomyces* strain was killer sensitive against the classical killer toxin.

On the other hand, when *S. cerevisiae* was seeded on *K. apiculata* lawn a zone of inhibition could be observed, however, since no ring of dead cells is present, cell death is absent, and the inhibition may be produced by metabolites other than yeast killer toxins.

In order to investigate the action of killer toxins in the conditions of pH and temperature occurring in the fermentation tanks, the test was realized also at 28°C. At this temperature the killing ability of strains was limited; only two positive killer yeasts (K1 and K10) on the sensitive strain, being K1 killer toxin more active than K10 toxin (data not shown).

Conclusion

In conclusion, the kinetic and metabolic behavior of *K. apiculata* mc1 and *Saccharomyces cerevisiae* mc2

Table 4 Killer activity or sensitive tests performed for tester strains with the reference strains at different medium pH and at 18°C

Tester strains	*S. cerevisiae* K1			*S. cerevisiae* K2			*C. glabrata* K4			*Kl. drosophilarum* K10			*K. apiculata* mc1			*S. cerevisiae* mc2		
	3.5	4.5	5	3.5	4.5	5	3.5	4.5	5	3.5	4.5	5	3.5	4.5	5	3.5	4.5	5
K. apiculata mc1	+	+	+	–	–	–	–	–	–	–	–	–				+	+	+
S. cerevisiae mc2	++	++	++	++	++	++	++	++	++	++	++	++	–	–	–			
S. cerevisiae[a] P351	++	++	++	++	++	++	++	++	++	++	++	++	–	–	–	–	–	–

[a] Sensitive strain

+ Inhibition

++ Inhibition with a blue periphery or zone of bluish-stained cells

– No effect

in pure and mixed culture, were examined. Even if both yeasts in mixed cultures reached lower maximal biomass, *K. apiculata* survive longer than in pure culture. It was observed that these yeasts produce higher level of acetic acid and ethyl acetate; however, undesirable production of volatile compounds was not detected in composite cultures. The existence of *K. apiculata* mc1, a non-*Saccharomyces* yeasts isolated from wine during alcoholic fermentation might be of technological interest. However, in wine biotechnology more specific information on the extent of its contribution is required.

Acknowledgments This work was supported by grants from Banco Rio, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT). Argentina

References

Amerine MA, Kunkee RE, Ough CS, Singleton VL, Webb AD (1980) The technolohy of wine making, 2nd edn. AVI Publishing, Westport

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

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

Ciani M (2001) Wine fermentation by multistarter inoculum. Industrie delle Bevande 30:35–39

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

Doi E, Shibata D, Matoba T (1981) Modified colorimetric ninhydrin methods for peptidase assay. Anal Biochem 118:173–184

Egli CM, Edinger 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

Fleet GH, Heard GM (1993) Yeasts: growth during fermentation. In: Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic, Chur, Switzerland, pp 27–54

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

Gardner N, Rodrigue N, Champagne CP (1993) Combined effects of sulfites, temperature and agitation time on production of glycerol in grape juice by *Saccharomyces cerevisiae*. Appl Environ Microbiol 59:2022–2028

Gil JV (1993) Influencia de cultivos iniciadores de levaduras en la fracción volátil de mosto de la cv. Monastrell, Tesis de Licenciatura, Facultad de CC Biológicas. Universidad de Valencia, Valencia

Ludovico P, Sousa MJ, Silva MT, Leao C, Corte-Real M (2001) *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology 147:2409–2415

Martini A (1993) Origin and domestication of wine yeast *Saccharomyces cerevisiae*. J Wine Res 3:165–176

Moreira N, Mendes F, Hogg T, Vasconcelos I (2005) Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. Int J Food Microbiol 103:285–294

Nissen P, Arneborg N (2003) Characterization of early deaths of non-*Saccharomyces* yeast in mixed cultures with *Saccharomyces cerevisiae*. Arch Microbiol 180:257–263

Pérez-Nevado F, Albergaria H, Hogg T, Girio F (2006) Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Sacchromyces* cerevisiae. Int J Food Microbiol 108:33–345

Povhe Jemec K, Cadez N, Zagorc T, Bubic V, Zupec A, Raspor P (2001) Yeast population dynamics in five spontaneous fermentations of Malvasia must. Food Microbiol 18: 247–259

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

Romano P, Fiore C, Paraggio M, Caruso M, Capece A (2003) Function of yeast species and strains in wine flavour. Int J Food Microbiol 86:169–180

Russel JA (1944) The colorimetric estimation of small amounts of ammonia by the phenol-hypoclorite reaction. J Biol Chem 156:457–461

Truong-Meyer X-M, Streihaiano P, Riba J-P (1997) Thermal inactivation of two yeast strains in a strawberry product: experimental data and kinetic model. Chem Eng J 65:99–104

Young TW, Yagiu M (1978) A comparison of the killer character in different yeasts and its classification. Antonie Van Leeuwenhoek 44:59–77

Zohre DE, Erten H (2002) The influence of *Kloeckera apiculata* and *Candida pulcherrima* yeasts on wine fermentation. Proc Biochem 38:319–324