A Method to Discriminate Between the *Candida stellata* and *Saccharomyces cerevisiae* in Mixed Fermentation on WLD and Lysine Agar Media

S. Di Maio[1], G. Polizzotto[1,2], D. Planeta[2*], D. Oliva[1]

(1) Istituto Regionale della Vite e del Vino (IRVV), Regione Siciliana, Via Libertà 66, 90143 Palermo, Italy
(2) Dipartimento di Ingegneria e Tecnologie Agro-Forestali - Sezione Industrie - Università degli Studi di Palermo, Viale Scienze n. 11, 90128 Palermo, Italy

Submitted for publication: June 2010
Accepted for publication: August 2010

Key words: Cycloheximide, *Candida stellata*, *Saccharomyces cerevisiae*, mixed fermentation

This paper presents a simple method to distinguish between *Candida stellata* and *Saccharomyces cerevisiae* yeasts during microbiological analyses. The method is based on differential yeast growth on a medium containing cycloheximide and a medium containing lysine as only nitrogen source (lysine agar). The cycloheximide resistance of 45 yeast strains belonging to *Candida stellata*, *Hanseniaspora uvarum, Hanseniaspora guilliermondii, Metschnikowia pulcherrima*, *Torulaspora delbrueckii, Zygosaccharomyces bailii, Kluyveromyces thermotolerans* and *Zygoascus hellenicus*, and 14 strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* on WL nutrient agar, was assayed. Cycloheximide resistance is characteristic of the species *H. uvarum, H. guilliermondii* and *Z. hellenicus*, while for the other yeasts it depends on the strain and the concentration of cycloheximide used. Two mg/L of cycloheximide allows selective counting of a strain of *C. stellata* (*Cs*3) compared to one of the sensitive *S. cerevisiae* strain (NDA21). Similar results can be obtained on lysine agar, but counts are reliable only with the additional spreading of a monolayer of *Saccharomyces* cells. The different cycloheximide resistance of *C. stellata* and *S. cerevisiae* can be used in the microbiological analysis of mixed cultures to monitor the individual growth of the two yeast species. This method can be applied to the study of mixed fermentations with other non-*Saccharomyces* species. The modified use of lysine agar is useful to a certain extent in the distinction of multistarter yeasts from the indigenous yeasts.

INTRODUCTION

In recent years, many researchers have focused their attention on studying the potential oenological use of non-*Saccharomyces* yeasts that are naturally present in musts and that are capable of producing secondary compounds that influence the organoleptic quality of wine as a result of their peculiar metabolic activities (Fleet, 2003; Romano *et al*., 2003). The use of some non-*Saccharomyces* yeasts was also investigated in experimental winemaking and in some cases the results are so encouraging that consideration for their application in oenology is increasing. Analysing the ability of the indigenous yeasts to carry out pure fermentation of the Macabeo variety Spanish grape, Clemente-Jimenez *et al*. (2004) identified strains of *Candida stellata* that were able to achieve complete fermentation, leaving little residual sugar and producing ethanol typical of table wines. They also showed that strains of *Pichia fermentans* and *Hanseniaspora uvarum* were able to produce large quantities of glycerol, even if distinguished by lower fermenting activity. The same authors also described wines characterised by a well-balanced composition of higher alcohol, also produced with fermentation by *H. uvarum, Issatchenkia orientalis* and *C. stellata* strains. Higher glycerol quantities have been produced in wines fermented by *C. stellata*, but with lower fermentation activity of the strains and an abundance of produced acetaldehyde, acetoin and higher alcohols (Ciani & Ferraro, 1996).

However, the reduced fermentation activity widespread among these non-*Saccharomyces* yeasts and the excessive production of off-flavour compounds do not permit the use of most non-*Saccharomyces* yeasts as a starter in pure fermentations. A new trend in oenological experimentation foresees non-*Saccharomyces*/*Saccharomyces* mixed inocula in must, with promising results (Jolly *et al*., 2006). Mixed preparations of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae* (www.chr-hansen.com) and *Torulaspora delbruekii, K. thermotolerans* and *S. cerevisiae* (www.chr-hansen.com) are already on sale for the production of wine with richer aroma. Much research on mixed fermentations has been dedicated to the *C. stellata/S. cerevisiae* yeasts for their ability to increase the wine's glycerol content and for their particular fructophilic properties (Ciani & Ferraro, 1998; Ciani & Fatichenti, 1999), their ability to generate a positive impact on the aromatic complexity of the wine (Scanes *et al*., 1998) and their ability to reduce the risk of stuck fermentation (Santos *et al*., 2008). Ciani

*Corresponding author: planeta@unipa.it

and Ferraro (1998) carried out *C. stellata/S. cerevisiae* mixed fermentations, following sequential inocula, and produced wines richer in glycerol and succinic acid and with less acetic acid and alcohol than that produced by *S. cerevisiae* alone. Later, Ferraro *et al*. (2000), in a similar fermentation, produced wines with a lower alcohol content than wines fermented by *S. cerevisiae* alone.

The sensory profiles of wines produced by *C. stellata*/*S. cerevisiae* mixed fermentation were determined by Soden *et al*. (1998, 2000) and the wine obtained by sequential two-yeast inoculum was enriched with the aroma of banana, flowers and lime, compared to the mono-inoculated *S. cerevisiae* control.

These data suggest that a great complexity and diversity in wines can be obtained with a industrial process when *C. stellata* and *S. cerevisiae* yeasts are selected appropriately. The frequent use of selected yeast strains in winemaking and the tendency to experiment with mixed yeast fermentations require an easy and fast distinction between inoculated yeasts and those that are naturally present in the musts and the possibility of control over the individual growth of various starter yeasts. Currently, different culture agar media are used, either selective or differential, which allows some discrimination between the different microorganism species involved in a wine fermentation. WL nutrient agar (Cavazza *et al*., 1992; Pallmann *et al*., 2001; Romancino *et al*., 2008), YM agar (Soden *et al*., 2000; Moreira *et al*., 2005) and YEPD (Ciani *et al*., 2006; Perez-Nevado *et al*., 2006) media are generally used to count total yeasts present in an oenological sample. The same media appropriately modified by the addition of an antimycotic or antiseptic (Moreira *et al*., 2005; Perez-Nevado *et al*., 2006) are selective and allow the counting of resistant yeasts. Instead, lysine agar (Morris & Eddy, 1957; Fowell, 1965; Heard & Fleet, 1986; Cavazza *et al*., 1992; Soden *et al*., 2000; Ciani *et al*., 2006) allows the counting of all the yeasts that are able to use lysine as the only nitrogen source. It follows that two medium types (selective and non-selective) are generally used together, as the information provided is complementary and allows the researchers to trace in some detail the fermentation trend. However, some selected media show an area of application that is restricted to only a few yeast species or genera (Moreira *et al*., 2005; Perez-Nevado *et al*., 2006), while when there are $< 10^4$ *Saccharomyces* cells/plate on lysine agar the individual cells develop into small colonies that may be mistaken for wild yeast colonies (Morris & Eddy, 1957; Fowell, 1965). Cavazza and Poznansky (1998) reported that *C. stellata* species grow on WL with a morphological distinction, grow on lysine agar and also on WL differential (WLD) equal to WL medium but containing cycloheximide, a yeast inhibiter active on a large number of yeast species. Thus, given the acquired importance in oenology of the *C. stellata/S. cerevisiae* mixed fermentations, in this study we have examined a method of investigation that uses the data collected on different culture media, including modified ones, to accurately determine the concentration of the various yeasts present in the analysed sample at each fermentation stage. In particular, the usability of this method was evaluated during the microbiological analysis on plates of a *C. stellata* strain from the yeast collection of the Sicilian Istituto Regionale della Vite e del Vino (IRVV) together with a commercial *S. cerevisiae* strain.

MATERIALS AND METHODS

Yeast strains

The yeasts used in this study belong to various genera and species: 14 *Saccharomyces* strains, including some belonging to different collections and most from commercial origins, as shown in Table 1; 42 non-*Saccharomyces* yeast isolates from the IRVV collection (Romancino *et al*., 2008), including 14 *Candida stellata* (*Cs*1-*Cs*14), four *Hanseniaspora guilliermondii* (*Hg*1-*Hg*4), five *H. uvarum* (*Hu*1-*Hu*4 and *Hu*03), three *Issatchenkia terricola* (*It*1-*It*3), four *K. thermotolerans* (*Kt*1-*Kt*4), three *Metschnikowia pulcherrima* (*Mp*1-*Mp*3), three *T. delbrueckii* (*Td*1-*Td*3), three *Zygosaccharomyces bailii* (*Zb*1-*Zb*3) and three *Zygoascus hellenicus* (*Zh*1-*Zh*3); and finally the *C. stellata* 6714, *H. guilliermondii* 6796 and *K. thermotolerans* 6232 strains of the University of Perugia's Industrial Yeasts Collection DBVPG (www.agr.unipg.it/dbvpg). The commercial *S. cerevisiae* NDA21 yeast was also selected by the IRVV for the production of high quality wines (Di Maio *et al*., 2006; Oliva *et al*., 2006). The isolation and oenological study of the strain *S. cerevisiae* A3-2 also took place in the same selection programme (Di Maio *et al*., 2009). The *Saccharomyces* yeasts were cultivated in agar-slant tubes containing Sabouraud dextrose agar (Oxoid), added with 10 g/L yeast extract (Oxoid) and maintained at 4°C. The non-*Saccharomyces* yeasts were cultivated in agar-slant tubes containing malt agar medium (malt extract 30 g/L, bacteriological agar 15 g/L) and maintained at 4°C. All the cultures were renewed periodically.

Plate screening of wine yeasts' cycloheximide resistance on WLD medium

A loop of yeast fresh cultures, cultivated on YPD agar (yeast extract 10 g/L, dextrose 20 g/L, peptone 20 g/L, agar 20 g/L), was smeared on WL nutrient agar (Oxoid) containing increasing cycloheximide concentrations: 0.5, 1.0, 2.0, 4.0 and 10.0 mg/L. The WL medium was prepared following the supplier's instructions, while the WLD medium was prepared as described

TABLE 1
List of *Saccharomyces* strains used in the cycloheximide resistance experiment.

Strain	Yeast	Source
A3-2	*S. cerevisiae*	IRVV
6167	*S. cerevisiae*	Diproval
L404	*S. cerevisiae*	Diproval
11719	*S. bayanus*	Diproval
12233	*S. bayanus*	Diproval
ICV-D254	*S. cerevisiae*	Lallemand
EC1118	*S. cerevisiae*	Lallemand
QD145	*S. cerevisiae*	Lallemand
Uvaferm43	*S. cerevisiae*	Lallemand
NDA21	*S. cerevisiae*	Biospringer
CKS-102	*S. cerevisiae*	Biospringer
UCLM-S325	*S. cerevisiae*	Biospringer
Fermol Arome Plus	*S. cerevisiae*	Pascal Biotech
Zymaflore F10	*S. cerevisiae*	Laffort

by Cavazza *et al*. (1992), adding cycloheximide to WL medium. The cycloheximide solution was prepared in absolute ethanol (99.8%, v/v) and added to the medium just before the distribution in the Petri dishes. The same medium but without antimycotic was used as a positive control for yeast growth. Yeasts were grown for three to six days at 28°C. The growth rate with cycloheximide was evaluated by comparing the growth of yeast in the medium with and without antimycotic. Growth on WLD equal to that in WL was considered positive (+) and showed the yeast's ability to grow in the given cycloheximide concentration; no growth was considered negative (-) and showed the yeast's inability to tolerate that concentration of antimycotic. Intermediate growth (-+, -/-+) was indicative of some yeast sensitivity to cycloheximide. The minimum inhibitory concentration (MIC) of cycloheximide was the lowest antimycotic concentration in which the yeast cells did not grow.

This assay was carried out on 14 yeast strains belonging to the *Saccharomyces* genus from various collections or the industry, and on 45 non-*Saccharomyces* yeasts belonging to the following species: *C. stellata, H. guilliermondii, H. uvarum, I. terricola, K. thermotolerans, M. pulcherrima, T. delbrueckii, Z. bailii* and *Z. hellenicus* (see Table 2). The *S. cerevisiae* NDA21 and *H. uvarum Hu*03 strains were used as references for growth in cycloheximide, because the first was sensitive and the second resistant to the antimycotic (Cavazza *et al*., 1992). All tests were carried out in triplicate.

TABLE 2
Cycloheximide (cyc) resistance of 14 *Saccharomyces* strains and 45 non-*Saccharomyces* yeasts. NDA21 and *Hu*03 (underlined) were used as reference of growth in cyc on WL and WLD with different antimycotic concentrations.

Yeast species	Yeast	Strain number	0.0 mg/L cyc	0.5 mg/L cyc	1.0 mg/L cyc	2.0 mg/L cyc	4.0 mg/L cyc	10.0 mg/L cyc
S. cerevisiae	NDA21, A3-2	2	+	+	-+	-	-	-
	6167	1	+	-+	-	-	-	-
	L404	1	+	+	+	-	-	-
	ICV-D254	1	+	+	-+	-	-	-
	EC1118	1	+	+	+	-+	-	-
	QD145, Uvaferm43, CKS-102, UCLM-S325, Fermol Arome Plus, Zymaflore F10	6	+	+	+	-	-	-
S. bayanus	11719	1	+	-	-	-	-	-
	12233	1	+	-+	-	-	-	-
H. guilliermondii	6796, *Hg*1 to *Hg*4	5	+	+	+	+	+	+
H. uvarum	*Hu*03, *Hu*1 to *Hu*4	5	+	+	+	+	+	+
I. terricola	*It*1, *It*2	2	+	+	+	+	-	-
	*It*3	1	+	+	+	-	-	-
K. thermotolerans	6232	1	+	-	-	-	-	-
	*Kt*1, *Kt*2, *Kt*4	3	+	+	-	-	-	-
	*Kt*3	1	+	-+	-	-	-	-
M. pulcherrima	*Mp*1 to *Mp*3	3	+	+	+	+	-+	-
T. delbrueckii	*Td*1, *Td*3	2	+	+	-	-	-	-
	*Td*2	1	+	+	+	-	-	-
Z. bailii	*Zb*1	1	+	+	+	-	-	-
	*Zb*2, *Zb*3	2	+	+	+	-+	-	-
Z. hellenicus	*Zh*1 to *Zh*3	3	+	+	+	+	+	+
C. stellata	*Cs*1, *Cs*6, *Cs*10, *Cs*13, *Cs*14	5	+	+	+	+	+	+
	*Cs*2 to *Cs*5, *Cs*7 to *Cs*9, *Cs*11, *Cs*12	9	+	+	+	+	+	-/-+
	6714	1	+	+	-+	-	-	-

Cycloheximide resistance of pure and mixed plating of *Cs*3 and NDA21 strains on WLD medium

Equal volumes of pure cultures of *C. stellata Cs*3 and *S. cerevisiae* NDA21 strains grown in YPD (yeast extract 10 g/L, dextrose 20 g/L, peptone 20 g/L) were mixed to obtain a mixture of yeast cells with a concentration corresponding to that of the relative pure culture for each yeast. The mixture and pure cultures were analysed microbiologically following the method described by Cavazza and Poznansky (1998), with some variations: for the three samples, 0.2 mL of 10^{-4}, 10^{-5} and 10^{-6} serial dilutions in sterile water were spread on WL and WLD with increasing cycloheximide concentrations of 2.0, 4.0 and 10.0 mg/L. The media were prepared as described in the previous section. The inocula were then incubated at 28°C for five to six days and the colony forming units (cfu) grown in the various media were counted. The cfus of *C. stellata* and *S. cerevisiae* yeasts were identified by macroscopic examination of the morphology and colour of the colonies, as described by Cavazza *et al*. (1992), Cavazza & Poznansky (1998) and Pallmann *et al*. (2001). Only data of plates containing between 25 and 250 cfu/plate were considered significant in the counting (Busta *et al*., 1984). The reported results are averages obtained from three different plating with repeatability within 7.7% (Cavazza & Poznansky, 1998).

Selective counting of pure and mixed plating of *Cs*3 strain on WLD and lysine agar media

The experiment was carried out as described in the previous section, but spreading dilutions of pure cultures and mixture of the *Cs*3 and NDA21 strains on WL and on WLD media with 2 mg/L cycloheximide and on lysine agar, in duplicate. The WL and WLD media were prepared as described in the previous section. The lysine agar (Oxoid) medium was prepared following the supplier's instructions. Only on lysine agar medium, 0.1 mL of pure culture of the L404 strain (Diproval) grown for 12 h in 1 mL of YPD, previously centrifuged (1500 x g, 5 min, 4°C) and resuspended in the same volume of sterile water, was widespread on the plates in addition to the 0.2 mL dilution to achieve a concentration of *Saccharomyces* higher than 10^4 cells/plate. The inocula were then incubated at 28°C and finally the cfus grown in the different media were counted. On the WL and WLD media the colonies of *C. stellata* and *S. cerevisiae* yeasts were identified by macroscopic examination of the morphology and colour, as previously described. The colonies of non-*Saccharomyces* yeast grown in the lysine agar medium on a monolayer of *Saccharomyces*, formed by pinpoint colonies, were counted. Only data of plates containing between 25 and 250 cfu/plate were considered significant in the counting (Busta *et al*., 1984). The reported results are averages obtained from three different plating with repeatability within 7.7% (Cavazza & Poznansky, 1998).

***Cs*3 fermentations in grape must**

Alcoholic fermentations were performed in 2009 vintage from Merlot grapes cultivated in a vineyard situated in Roano county (Palermo, Italy). The grapes were pressed and the must (24.45°Brix, pH 3.55) was sulphited (0.05 g/L). The must was subdivided into two aliquots of 100 L, each one inoculated (6×10^6 cfu/mL) with *Cs*3 *C. stellata* liquid culture grown in sterile must (20°Brix, pH 3.20). Fermentations lasted 13 days

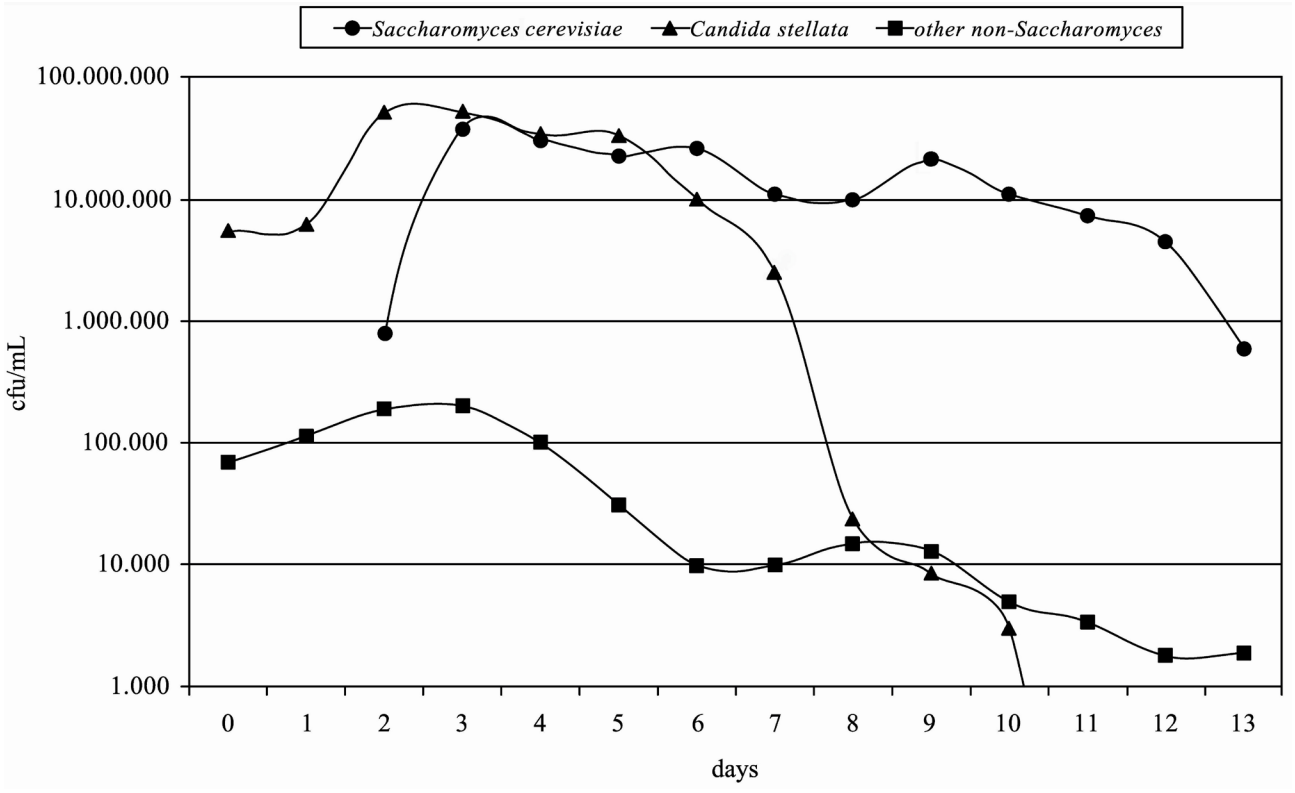

FIGURE 1

Growth curves of *Candida stellata, Saccharomyces cerevisiae* and other non-*Saccharomyces* yeasts in a grape must inoculated with *Cs*3 strain.

TABLE 3
Results of pure and mixed plating of *C. stellata Cs*3 (IRVV) and *S. cerevisiae* NDA21 (Biospringer) strains spread on WL and WLD with different cycloheximide (cyc) concentrations.

Culture	cfu/mL on 0 mg/L cyc	cfu/mL on 2 mg/L cyc	cfu/mL on 4 mg/L cyc	cfu/mL on 10 mg/L cyc
*Cs*3	150 x 10^6	160 x 10^6	160 x 10^6	0
NDA21	50 x 10^6	0	0	0
*Cs*3 + NDA21	220 x 10^6	150 x 10^6	150 x 10^6	0

TABLE 4
Results of pure and mixed plating of *C. stellata Cs*3 (IRVV) and *S. cerevisiae* NDA21 (Biospringer) strains spread on lysine agar (LA), WL and WLD with 2 mg/L cycloheximide (cyc).

Yeast strain	cfu/mL on LA	cfu/mL on WL	cfu/mL on WLD 2 mg/L cyc
*Cs*3	380 x 10^6	360 x 10^6	350 x 10^6
NDA21	0	190 x 10^6	0
*Cs*3 + NDA21	370 x 10^6	520 x 10^6	350 x 10^6

at 25°C. Daily microbiological analyses were performed on WL, WLD (2 mg/L cycloheximide) and lysine agar (Oxoid), as previously described: data referred to in Figure 1 as "other non-*Saccharomyces* yeasts" were obtained as differences between the counting results in lysine agar and *Candida* cfus counted on WLD.

The analysis of length of the restriction fragments of polymorphisms of yeast mitochondrial DNA (mtDNA-RFLP) was performed on 50 *C. stellata* cfus from WLD plates on the fifth fermentation day. Yeast DNA was extracted as described by Querol *et al*. (1992). The total yeast DNA was digested with restriction endonuclease HpaII (Biolabs), according to the supplier's instructions (Pramateftaki *et al*., 2000). The molecular fragments were separated through horizontal electrophoresis on agarose gel at 0.7% (w/v) in 0.5 x TBE (45 mmol Tris base, 45 mmol boric acid, 1 mmol EDTA pH 8.0), using 1 kb DNA ladder (Promega) as a marker of molecular weight. At the end of the run, the gel was stained with ethidium bromide (0.5 µg/mL) and UV transilluminated. The fluorescent image was acquired using a Gel Doc 2000 system (Bio-Rad Laboratories) running Quantity One software (Bio-Rad Laboratories).

Similar data were obtained in both fermentations. The reported data (Fig. 1) refer only to one.

RESULTS AND DISCUSSION

Cycloheximide resistance of yeasts

The aim of this experiment was to evaluate the cycloheximide resistance of various yeast species (Table 2). Although *C. stellata* is generally considered a negative-cycloheximide species, for the 14 strains of *C. stellata* of the IRVV collection our results are partly in agreement with Cavazza and Poznansky (1998), who reported that *C. stellata* is able to grow up to 4 mg/L cycloheximide. However, although these authors advise increasing the amount of cycloheximide in the medium up to 10 mg/L to prevent the growth of *C. stellata*, five IRVV strains were able to grow in this antimycotic concentration (Table 2). The *C. stellata* DBVPG 6714 strain, used as a reference from the other collection, shows contrasting behaviour, being sensitive to the lower amount of cycloheximide used in the experiment, equal to 0.5 mg/L.

The results obtained for the 14 *Saccharomyces* strains analysed in this study, belonging to the *S. cerevisiae* and *S. bayanus* species, confirm the effective sensitivity of these species to the antimycotic (Cavazza *et al*., 1992; Cavazza & Poznansky, 1998). The *S. bayanus* 11719 strain was the most sensitive and was unable to grow even in the lowest antimycotic concentration that was used (0.5 mg/L). The *S. cerevisiae* EC1118 strain was the only analysed *Saccharomyces* able to growth in 2 mg/L cycloheximide, although this growth was reduced. The control strain *S. cerevisiae* NDA21, as expected, was extremely sensitive to the antimycotic, and its growth was already partially inhibited at a concentration of 1 mg/L. The sensitivity to cycloheximide is spread throughout the species, but it is not characteristic of the whole *Saccharomyces* genus. Deak and Beuchat (1996) report that the species *S. dairenensis* and *S. exiguus* possess some degree of resistance to 100 mg/L cycloheximide. The MIC of *S. cerevisiae* and *S. bayanus* yeasts used in this experiment therefore corresponds to 2 mg/L on WL medium, because in such a concentration none of these yeasts is able to grow as in medium without antimycotic.

All the analysed *H. uvarum* and *H. guilliermondii* yeasts are resistant to the highest used cycloheximide concentration, equal to 10 mg/L. The species *H. guilliermondii*, *H. uvarum* and *H. valbyensis* have also been reported to be able to grow in up to 100 mg/L cycloheximide, while *H. osmophila* is unable to grow at such a high antimycotic concentration (Deak & Beuchat, 1996). The three analysed *Z. hellenicus* grew in 10 mg/L cycloheximide (Table 2), confirming the resistance of *Z. hellenicus* to this antimycotic (Prasad *et al.*, 2005). Lower cycloheximide resistance is shown by *M. pulcherrima, Z. bailii* and *T. delbrueckii* yeasts, with values that are often different according to the strain, but always lower or equal to 2 mg/L: these results agree with those of Cavazza *et al*. (1992). Nevertheless, we noted partial growth of *M. pulcherrima* in 4 mg/L antimycotic. Furthermore, we report similar results for *I. terricola* and *K. thermotolerans* (Table 2). Thus, the MIC of cycloheximide relating to the analysis of these species is 2 mg/L, with some reservations only for *M. pulcherrima*. Other *Zygosaccharomyces* yeasts, such as *Z. fermentatii* and *Z. florentinus,* grow up to 100 mg/L (Deak & Beuchat, 1996).

Cycloheximide resistance during microbiological analysis of pure and mixed plating of *Cs*3 and NDA21 strains

The aim of this experiment (Table 3) was to distinguish between *S. cerevisiae* and *C. stellata* in mixed plating on the basis of their different cycloheximide resistance. The cfu counts of the *C. stellata Cs*3 and *S. cerevisiae* NDA21 strains, grown on different media, fully confirmed the data of the previous section. A pure culture of the *Cs*3 strain was able to give comparable

concentrations in medium without or with 2 and 4 mg/L cycloheximide (150 to 160 x 10^6 cfu/mL), showing that these antimycotic concentrations do not cause significant changes in *Cs*3 growth. On the other hand, just visible and indistinguishable pinpoint cfus grew in 10 mg/L cycloheximide, but were unable to grow further: growth was therefore considered null. The *Cs*3 colony morphology was that described by Cavazza and Poznansky (1998), namely "colonies of a pea green colour, which over time become darker at the centre, while the edges are clear, and rising to a dome or umbo", and did not change in shape and/or colour at 2 and 4 mg/L cycloheximide. However, a delay in the colonies' growth was noted at these cycloheximide concentrations compared to the medium without antimycotic: therefore, the colonies' sizes were different in the various media on the same incubation day and the counting of the colonies required one or two more incubation days than those reported by Cavazza and Poznansky (1998). Extending the incubation time in 10 mg/L cycloheximide medium showed no late growth of the *Cs*3 yeast. Conversely, a pure culture of NDA21 strain that was reported to be able to grow on WL "with circular colonies also of considerable diameter and colour varying from cream to green, with domed shape, opaque smooth surface and creamy consistency" (Cavazza *et al*., 1992), did not grow at any cycloheximide concentration. In the mixed plating of the two yeasts on WL without antimycotic, the *C. stellata* and *S. cerevisiae* colonies were easily distinguished by their different morphology; only *C. stellata* morphology was found on the 2 and 4 mg/L cycloheximide media, with an increasing delay in growth at rising antimycotic concentrations.

Selective counting of *Cs*3 yeast during microbiological analysis of pure and mixed plating of *Cs*3 and NDA21 strains

In this experiment we used lysine agar, WL and WLD (2 mg/L cycloheximide) for collecting as much data as possible about mixtures of *S. cerevisiae* and *C. stellata*. Table 4 shows the cfu/plate data obtained by the spreading of *Cs*3 and NDA21 liquid cultures and from a corresponding mixture of the two strains. The pure NDA21 culture grew only on WL medium. In contrast, the pure *Cs*3 culture grew comparably also in lysine agar and WLD (Table 4), and similar concentrations of *Cs*3 were found when it was plated together with NDA21. However, in the plating of mixed cultures on lysine agar it was necessary to co-inoculate a high number of another *Saccharomyces* strain (> 10^4 cells/plate), because below this value this species produces colonies that can wrongly be considered as non-*Saccharomyces* (see Materials and Methods): *Candida* colonies grown on lysine agar were easily countable on the *Saccharomyces* monolayer.

Selective counting of *Cs*3 strain during microbiological analysis of wine fermentations

The use of WL, WLD (2 mg/L cycloheximide) and lysine agar makes it possible to monitor the growth of a strain of *C. stellata* in the presence of *S. cerevisiae* and other wine yeasts. Figure 1 shows the growth curves of *C. stellata*, *S. cerevisiae* and other non-*Saccharomyces* yeasts in a grape must inoculated with the *Cs*3 strain. All the non-*Saccharomyces* yeasts, *C. stellata* included, were able to grow on lysine agar, but *C. stellata* was identified on WL and WLD by colony morphology. On WL all the other yeasts were also identifiable by different colony morphologies, at least while the *S. cerevisiae* colonies were not so abundant as to make it impossible to recognise any other species. Only genus *Hanseniaspora* was found on WLD together with *C. stellata*, confirming that this genus is widespread in the early stages of the spontaneous fermentation of Sicilian grapes (Romancino *et al*., 2008): their different morphologies allowed their easy and rapid distinction during the counting of the colonies. From the second day, wild *Saccharomyces* yeasts were found on WL and, as expected, they did not grow on WLD and lysine agar. From the third fermentation day onwards, *Saccharomyces* yeasts grew increasingly numerously on WL medium, preventing the correct counting of colonies of any other yeast species in this medium, although *C. stellata* colonies were counted on WLD. From the plating on lysine agar it was possible to collect the data of all the non-*Saccharomyces* yeasts, even in the early days of fermentation in the presence of a low number of *S. cerevisiae* cfus, thanks to the *Saccharomyces* monolayer. mtDNA-RFLP analyses demonstrated the massive presence (92 to 100%) of the *Cs*3 strain in the *C. stellata* population of the fermenting must (data not shown).

CONCLUSIONS

C. stellata clearly is able to grow at low cycloheximide concentrations, but with important strain-dependent differences: for the *C. stellata* IRVV strains, which showed a higher resistance compared to the available data (Cavazza & Poznansky, 1998), this phenotypical evidence is a probable sign of their common geographical origin (Romancino *et al*., 2008). Indeed, studies on *S. cerevisiae* cycloheximide resistance show that mutations in different genetic loci can induce this antimycotic resistance (Del Pozo *et al*., 1991; Mortimer *et al*., 1991). These species- and strain-specific differences in cycloheximide resistance make it possible to use an appropriate WLD as tool for monitoring *C. stellata* strains in all the stages of mixed fermentations. Furthermore, the use of WLD medium has the advantage of distinguishing the cfu morphology of different species and allowing a further check on data obtained from other selective and non-selective media. We demonstrated that it is possible to collect data about a single *C. stellata* strain in a fermentation with *Saccharomyces* and non-*Saccharomyces* yeasts using WL, WLD and lysine agar. Furthermore, this method has the potential to distinguish between *C. stellata* and *K. thermotolerans*, both generally found in grape musts, growing on WL with very similar morphologies (Romancino *et al*., 2008) but showing very different levels of resistance to cycloheximide. Finally, this method allows the distinction between inoculated and wild *C. stellata,* if coupled with molecular analyses of *C. stellata* strains (Pramateftaki *et al*., 2000).

LITERATURE CITED

Busta, F.F., Peterson, E.H., Adams, D.M. & Johnson, M.G., 1984. Colony count methods. In: Speck, M.L. (ed). Compendium of methods for the microbiological examination of foods. Washington, DC. pp. 62–83.

Cavazza, A. & Poznansky, E., 1998. Le analisi microbiologiche nel laboratorio enologico. Vignevini 6, 42-53.

Cavazza, A., Grando, M.S. & Zini, C., 1992. Rilevazione della flora microbica di mosti e vini. Vignevini 9, 17-20.

Ciani, M. & Fatichenti, F., 1999. Selective sugar consumption by apiculate yeasts. Lett. Appl. Microbiol. 28, 203-206.

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 improve the quality of wines. J. Appl. Microbiol. 85, 247-254.

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

Clemente-Jimenez, J.M., Mingorance-Carloza, L., Martìnez-Rodrìguez, S., Las Heras-Vàzquez, F.J. & Rodrìguez-Vico, F., 2004. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiol. 21, 149-155.

Deak, T. & Beuchat, L., 1996. Handbook of food spoilage yeasts. CRC Press, pp. 143-150, Buca Raton, FL.

Del Pozo, L., Abarka, D., Claros, M.G. & Jimènez, A., 1991. Cycloheximide resistance as a yeast cloning marker. Curr. Genet. 19, 353-358.

Di Maio, S., Polizzotto, G., Notarbartolo, G., Planeta, D., Genovese, S., Gurrieri, C. & Oliva, D., 2006. Isolamento e caratterizzazione di un nuovo ceppo di lievito di interesse enologico. L'Enologo 11, 93-98.

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G. & Oliva. D., 2009. Studio delle popolazioni di *Saccharomyces* indigeni in antiche cantine della Sicilia sud-orientale. www.infowine.com – Rivista Internet di Viticoltura ed Enologia n. 9/2, 1-13.

Ferraro, L., Fatichenti, F. & Ciani, M., 2000. Pilot scale vinification process using immobilizer *Candida stellata* cells and *Saccharomyces cerevisiae*. Process. Biochem. 35, 1125-1129.

Fleet G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86, 11-22.

Fowell, R.R., 1965. The identification of wild yeast colonies on Lysine Agar. J. Appl. Bacteriol. 28, 373-383.

Heard, G.M. & Fleet, G.H., 1986. Evaluation of selective media for enumeration of yeasts during wine fermentation. J. Appl. Bacteriol. 60, 477-481.

Jolly, N.P., Augustyn, O.P.H. & Pretorius, I.S., 2006. The role and use of non-*Saccharomyces* yeasts in wine production. S. Afr. J. Enol. Vitic. 27, 15-39.

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.

Morris, E.O. & Eddy, A.A., 1957. Method for the measurement of wild yeast infection in pitching yeast. J. Inst. Brew. 63, 34-43.

Mortimer, R.K., Schild, C.R., Contopoulou, C.R. & Kans, J.A., 1991. Genetic and physical maps of *Saccharomyces cerevisiae*. In: Guthrie, C. & Fink, G.R. (eds). Methods in enzymology: guide to yeast genetics and molecular biology. Academic Press, pp. 827-863, NY.

Oliva, D., Ragusa, M., Capraro, F., Genna, G., Prinzivalli, L., Sparacio, A., Sparla, S. & Melia, V., 2006. Produzione di vini rossi sperimentali con il ceppo di lievito *S. cerevisiae* NDA21. L'Enologo 11, 85-92.

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

Perez-Nevado, F., Albergaria, H., Hogg, T. & Girio, F., 2006. Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 108, 336-345.

Pramateftaki, P.V., Lanardis, P. & Typas, M.A., 2000. Molecular identification of wine yeasts at species or strain level: a case study with strains from two vine-growing areas of Greece. J. Appl. Microbiol. 89, 236-248.

Prasad, G.S., Maylraj, S., Nitu, S., Vijeyta, S., Kakoli, B. & Banwari, L., 2005. *Candida digboiensis* sp. nov., a novel anamorphic yeast species from an acidic tar sludge-contaminated oilfield. Int. J. Syst. Evol. Microbiol. 55, 967-972.

Querol, A., Barrio, E., Huerta, T. & Ramon, D., 1992. Molecular monitoring of wine fermentation conducted by active dry yeast strains. Appl. Environ. Microbiol. 58, 2948-2953.

Romancino, D., Di Maio, S., Muriella, R. & Oliva, D., 2008. Analysis of non *Saccharomyces* yeast populations isolated from grape must from Sicily (Italy). J. Appl. Microbiol. 6, 2248-2254.

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.

Santos, J., Sousa, M.J., Cardoso, H., Inàcio, J., Silva, S., Spencer-Martins, I. & Leão, C., 2008. Ethanol tolerance of sugar transport and the rectification of stuck wine fermentations. Microbiology 154, 422-430.

Scanes, K.T., Hohmann, S. & Prior, B.A., 1998. Glycerol production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review. S. Afr. J. Enol. Vitic. 19, 15-21.

Soden, A., Francis, I.L., Gockowiak, H., Lee, T.H. & Henschke, P.A., 1998. The use of non-*Saccharomyces* yeasts in winemaking. In: Blair, R.J., Sas, A.N., Hayes, P.F. & Høj, P.B. (eds). Proc. 10th Aust. Wine. Ind. Tech. Conf., August 1998, Sydney, Australia. pp. 166 – 171.

Soden, A., Francis, I.L., Oakey, H. & Henschke, P.A., 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust. J. Grape Wine Res. 6, 21-30.