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ORIGINAL ARTICLE

# Indigenous wine killer yeasts and their application as a starter culture in wine fermentation

**T. Zagorc[1], A. Maráz[2], N. Cadez[1], K. Povhe Jemec[1], G. Péter[3], M. Resnik[4], J. Nemanič[4] and P. Raspor[1,*]**

*Wine yeast strains were isolated from seven fermentations of the red wines 'Refošk' and 'Teran', produced in the southwestern part of Slovenia. Among 613 isolated yeast strains, 22 expressed killer activity against the supersensitive strain* Saccharomyces cerevisiae. *Killer strains were isolated at different stages of wine fermentation but did not dominate in any of them. Species identification was based on the combination of RFLP analysis of an amplified rDNA region and biochemical–physiological tests. Killer isolates were identified as* S. cerevisiae, Pichia anomala, Pichia kluyveri, Pichia pijperi, Hanseniaspora uvarum *and* Candida rugosa. *Electrophoretic karyotyping was used to differentiate strains of the same species. Fermentation properties of four* S. cerevisiae *strains that possessed stable killer activity were characterized in fermentations of Malvasia must by studying their population dynamics and chemical composition and by sensory analysis of the wines produced. In order to compare the results, spontaneous fermentations and fermentations induced by commercial yeast starters were performed concomitantly. The local killer strain $S_S$12/10 showed the best fermentation properties and produced wine with favourable characteristics.*



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*[1]University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Jamnikarjeva 101, 1000 Ljubljana, Slovenia*
*[2]Szent István University, Faculty of Food Science, Somlói út 14-16, 1118 Budapest, Hungary*
*[3]Szent István University, NCAIM, Somlói út 14-16, 1118 Budapest, Hungary*
*[4]Agricultural Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia*

## Introduction

The fermentation of grape juice into wine is a complex microbiological process involving interactions between yeasts, bacteria, fungi and their viruses. Because of their metabolic activity, yeasts play a central role in the must fermentation process. During natural fermentat-ion, many different yeast strains undergo sequential substitution. The inoculation with yeast starter cultures dramatically changes the microbiology of the wine fermentation process (Fleet and Heard 1993).

Some yeast strains secrete protein or glycoprotein toxins that are lethal to sensitive strains of different species and genera and have been designated as killer yeasts. Killer activity was found among the genera *Saccharomyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Kluyveromyces, Pichia, Williopsis* and *Zygosaccharomyces*. Killer yeast strains have been isolated from many different environmental niches (lakes, rivers, fruits and vegetables) as well as from the fermentation of various

*Corresponding author. Prof. Dr Peter Raspor, Fax: +386 1 2574092. E-mail: peter.raspor@bf.uni-lj.si

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foods and beverages. Ecological studies indicate that killer activity could be a mechanism of competition, with the production of a toxic compound by one yeast cell excluding others from its habitat (Starmer et al. 1987, Jacobs et al. 1991, Yap et al. 2000).

The genetic determinants of killer activity can be either chromosomal or extra chromosomal, in the form of linear DNA plasmids or double-stranded RNA virus-like particles. The killer phenotype in *Saccharomyces cerevisiae* is associated with the presence of two double-stranded RNA molecules: the M genome codes for toxin and immunity, while L dsRNA encodes the protein coat for both molecules (Tipper and Bostian 1984).

Several surveys have been conducted to verify the incidence of killer yeasts in spontaneous must fermentation. The studies have shown that killer yeasts are distributed differently in various wine-producing areas. The incidence of killer yeasts varies with respect to the fermentation stage and vintage period, differing between the first vintage and successive ones. Analysis of karyotypes usually shows a mixed killer population within a wine fermentation in which killer yeasts were present. The highest frequency of the killer phenotype has been found among strains *S. cerevisiae*. The distribution of killer strains is influenced by the pH value of the must (Kitano et al. 1984, Cansado et al. 1991, Vagnoli et al. 1993, Hidalgo and Flores 1994).

The enological interest in killer yeasts arises because these yeasts, when present, might dominate a wine fermentation. When a killer yeast possesses positive enological characteristics and is used as a starter culture, the wine produced can be excellent and the fermentation process 'self-protected'. When selecting killer yeast for a starter culture, it is important to test the strains in their natural environment (i.e. must/wine) since killer strains can show different characters in large-scale wine fermentation than in laboratory tests with artificial media (Silva 1996).

The aim of our work was to study indigenous wine killer yeasts: their distribution in selected red wine fermentations, their identity determined by a combination of molecular and classical methods, and their fermentation properties with potential use as starter cultures. The local strains used as starter cultures have already been the object of some fermentation studies (Regodon et al. 1997, Peŕez-Coello 1999). We performed fermentation experiments with Malvasia must either inoculated with selected indigenous killer yeasts or commercial starter yeasts, or fermented spontaneously in order to study microbiological, chemical and sensory properties of must/wine during the fermentation processes.

## Materials and Methods

### *Yeasts strains*

All strains are stored in the Culture Collection of Industrial Microorganisms (ZIM, Ljubljana).

### *Reference strains*

*Candida rugosa* var. *rugosa* CBS $613^T$, *Hanseniaspora uvarum* NRRL Y-1614, *Pichia anomala* NCAIM 1109, *P. kluyveri* var. *kluyveri* CBS 188, *S. cerevisiae* ATCC 18824, *S. cerevisiae* S6 (supersensitive strain for broad range killer activity), *S. cerevisiae* ATCC 42917 (K1), *S. cerevisiae* NCYC 738 (K2).

### *Yeast isolates*

Indigenous wine yeast strains (497 isolates) were isolated during fermentations of the red wines 'Teran' (wineries G, M and S) and 'Refošk' (wineries D, K and J). In winery S, spontaneous fermentation ($S_S$) and induced fermentation ($S_I$) were conducted concomitantly. The starter culture $S_I$ possessed a neutral phenotype. One hundred and sixteen yeast strains were isolated from a smear taken from the winery equipment. Samples were spread on YEPD agar plates (0·5% yeast extract, 0·5% peptone, 1% glucose, 2% agar) in different dilutions and incubated for 2 days at 25°C.

### *Assay of the killer phenotype*

YEPD agar containing 0·003% (w/v) methylenblue was buffered to pH 3·0, 4·2 and 4·7 with a

0·1 M citrate-phosphate buffer and inoculated with the sensitive strain *S. cerevisiae* S6. The yeast strains were streaked on top of the seeded YEPD agar. The plates were incubated at 20°C for 3 days (Stumm et al. 1977). Strains were designated as killers when a clear zone of inhibition margined by blue-coloured cells surrounded the inoculated strains. In interaction assays, the plates were seeded with killer isolates, and killer strains be-longing to type K1 and K2 were streaked on top.

### *dsRNA characterization*

Extraction of dsRNA was carried out by the method of Fried and Fink (1978). Samples of extracted nucleic acids were separated by 1·5% agarose gel electrophoresis at a constant current of 100 mA. The nature of the dsRNA bands was determined by the digestion of RNA by 1 μg ml$^{-1}$ RNase A (Sigma, St. Louis, USA).

### *Amplification of rDNA sequence*

Total DNA isolation of the strain was performed as described previously by Smole-Možina et al. (1997).

The DNA amplifications were performed in Perkin Elmer PCR System 2400. They were carried out in a 20 μl reaction volume containing 5–15 ng yeast DNA, 1 × PCR buffer, 20 μM each of dNTP, 2 mM $MgCl_2$, 20 pmol of each primer and 1 U of *Taq* DNA polymerase (Perkin Elmer, Wellesley, USA). The primer pair used for the amplification of the 18S-ITS1-5·8S-ITS2 rDNA sequence was: NS1 (5′ GTAGTCATATGCTTGTCTC 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White et al. 1990). Parameters of amplification were as follows: denaturation at 95°C for 2 min, followed by 35 cycles (30 s at 95°C, 30 s at 60°C and 3 min at 72°C) and by a final elongation step of 7 min at 72°C. PCR products were restricted with the endonucleases, *Hae*III, *Msp*I and *Rsa*I (Roche, Diagnostics, Mannheim, Germany). Restricted fragments were separated by electrophoresis in 1·5% agarose gels and 1 × TAE buffer, stained with ethidium bromide (0·5 μg m1$^{-1}$) and documented on Polaroid 667 film.

### *Karyotype analysis by pulsed field gel electrophoresis (PFGE)*

Yeast chromosomes were isolated by the method essentially described by Carle and Olson (1985) and modified by Raspor et al. (2000). Late logarithmic phase cultures were embedded in a low melting point agarose and sequentially digested with Novozym (Sigma) and Proteinase K (Roche, Diagnostics). The electrophoretic karyotyping was performed by the CHEF apparatus LKB-pulsaphor$^{TM}$ (Pharmacia LKB, Uppsala, Sweden). Yeast chromosomes were separated by electrophoresis using 1% agarose gels in a 0·5 × TBE buffer at 12°C. Conditions for electrophoresis of *Saccharomyces* strains were as follows: pulse times of 60 s for 15 h, 90 s for 8 h and 100 s for 1 h at 180 V, and for non-*Saccharomyces* strains: pulse times 150 s for 24 h, 300 s for 24 h, and 600 s for 20 h at 100 V. Gels were stained with ethidium bromide (0·5 μg ml$^{-1}$) and documented on Polaroid 667 film.

### *Biochemical and physiological tests used for identification*

Instructions for the preparation of materials, selection of tests and testing conditions were followed as described by Barnett et al. (1990). The results were evaluated by the PC program for yeast identification (Barnett et al. 1996).

### *Fermentation of Malvasia must*

*Experiment:* Nine small-scale fermentations were performed in glass fermenters containing 9 l of must. The initial pH value of the must was 3·28 and the concentration of sugars was 227 g l$^{-1}$. No sulfur was added to the must. Indigenous yeast strains, $S_S$7/11, $S_S$7/16, $S_S$12/10 and $S_I$7/9, were chosen as starters in four fermentations on the basis of their stable killer activity. Commercial starter yeasts, N96, NT7, VIN13 and NT45 (Anchor Yeast, Cape Town, South Africa), induced a further four fermentations of Malvasia must of the same origin. One sample of Malvasia must was left to ferment spontaneously.

*Inoculum:* Single colony cultures were cultivated in a YM medium (0·3% yeast extract, 0·3% malt extract, 1% glucose) until they

reached the late exponential phase. Cells were harvested by centrifugation and diluted for final concentrations in must $10^5$ cells $ml^{-1}$. Commercial starters were prepared according to the instructions of the producer and added in the same concentration to the must.

*Sampling:* Samples (40 ml) of Malvasia must/wine were taken from the center of the fermenter. The samples for chemical analysis were centrifuged and stored frozen. The results presented refer to the following sampling points: (i) must before the inoculation; (ii) end of fermentation (the sugar content remained constant). Samples (1 ml) were plated on YEPD agar plates in different dilutions. During nine fermentations, 1919 strains were isolated: must before inoculation (160), spontaneous fermentation A0 (120), four fermentations with local killer starters (852) and four fermentations with commercial killer starters (787). All isolates were tested for their killer activity using replica plating method. The isolates from the last stage of fermentation were also characterized by karyotyping.

### Chemical analyses

*Ethanol, methanol, higher alcohols and ethyl acetate* were determined by gas-chromatographic analyses, carried out using a Hewlett-Packard 5890 gas chromatograph. Wine samples (1 μl) with 2-methyl-2-propanol as an internal standard were injected directly. A HP-INNOWAX column (60 m × 0·25 mm with 0·25 μm film thickness) was used. The temperature programme was: 35°C/10 min, then 5°C/min to 100°C and, finally, 40°C/min to 200°C. Injector and detector temperatures were 200°C and 250°C, respectively. Hydrogen N-48 at 1 ml $min^{-1}$ was the carrier gas.

*Sugars and organic acids* were determined using a Hewlett-Packard 1100 liquid chromatograph with Bio-Rad HPX-87H column. The mobile phase used was 0·007 M $H_2SO_4$. Organic acids and sugars were detected by a UV detector and by a detector of the refraction index, respectively (OIV 1993).

*Total and free sulfur dioxides* were determined according to the methodology of OIV (1993).

*Sensory evaluation:* The sensory analysis of Malvasia wines was performed 6 months after the end of alcoholic fermentation by highly skilled wine tasters according to the Buxbaum method (details available at: http://www.hr/wine/cbuxbaum.html). Results were expressed in the grading of 0 to 20 points, where 20 points represented the best results. The assessment took place in standard sensory analysis chambers (ISO 8589).

## Results and Discussion

### Killer yeasts in the production of the red wines Teran and Refošk

Our study of killer yeast distribution was focused on the production of the red wines Refošk and Teran, produced in the SW area of Slovenia. The occurrence of killer yeast strains was followed in six wineries with long wine-making traditions. The production capacities of the individual wineries differed from small production for domestic use to market-sale production. Red wine technology differs from the production of white wines through a step involving the maceration of crushed grapes that occurs with extensive air and equipment contact. Therefore, a more heterogeneous population of yeasts can be expected.

During seven fermentations of Teran and Refošk, killer yeasts were found at 11 out of 28 sampling points (Table 1). Among the 497 yeast strains isolated during the fermentations, 19 strains expressed killer activity against the sensitive strain *S. cerevisiae* S6, in laboratory conditions. On the first day of maceration, eight killer strains were isolated. They all belonged to the non-*Saccharomyces* species and were identified as *Pichia anomala, Pichia kluyveri, Hanseniaspora uvarum* and *Candida rugosa*. The macerated grapes were pressed on day 7 of fermentation. At this stage, three killer strains were isolated in wineries D and $S_S$ and further three killer strains were isolated from the late fermentation phase in wineries D, K and $S_I$. All of them were identified as

**Table 1.** Frequency of killer strains among all isolates at four consecutive sampling times during the fermentation processes of Refošk and Teran wines

| Technological stage | Day | Refošk wines | | | Teran wines | | | |
|---|---|---|---|---|---|---|---|---|
| | | J | D | K | $S_S$ | $S_I$ | G | M |
| Crushing of grapes | 0 | 21/0* | 19/1 | 22/0 | 19/0 | 17/2 | 8/0 | 14/5 |
| Middle fermentation | 7 | 13/0 | 26/1 | 16/0 | 17/2 | 19/0 | 18/0 | 16/0 |
| Late fermentation | 10 | 20/0 | 23/1 | 19/1 | 20/0 | 19/1 | 16/0 | 20/0 |
| End of fermentation | 12 | 11/0 | 19/2 | 14/0 | 15/2 | 20/0 | 18/1 | 18/0 |
| Total | | 65/0 | 87/5 | 71/1 | 71/4 | 75/3 | 60/1 | 68/5 |

*First number indicates the total number of yeast isolates, while the second number that of killer strains.

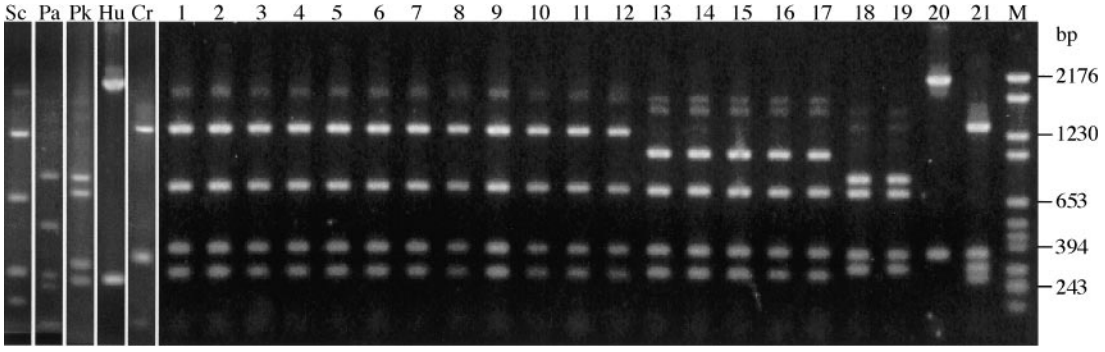


**Figure 1.** *Msp*I-RFLP of the amplified 18S-ITS1-5.8S-ITS2 sequence of killer isolates and the strains compared to RFLP-database of type strains; **Sc** *S. cerevisiae*, **Pa** *P. anomala*; **Pk** *P. kluyver*; **Hu** *H. uvarum*; **Cr** *Candida rugosa*, **1.** $S_S$7/11; **2**. $S_S$7/16; **3.** $S_S$12/10; **4.** $S_S$12/11; **5.** D8/5; **6.** D10/4; **7.** SK1; **8.** SK2; **9.** SK3; **10.** $S_I$7/9; **11.** K8/4; **12.** G12/6; **13.** M1/7b; **14.** M1/9; **15.** M1/10; **16.** M1/11; **17.** M1/12; **18.** D1/5; **19.** D12/1; **20.** $S_I$1/3; **21.** $S_I$1/8; **M.** marker.

*S. cerevisiae*. At the end of fermentation, on the 12$^{th}$ day, five killer strains were present and identified as *P. pijperi, P. kluyveri* and *S. cerevisiae*.

Rapid molecular identification of the killer strains was performed by restriction fragment length polymorphism (RFLP) analysis of amplified 18S-ITS1-5.8S-ITS2 rDNA regions (Guillamón et al. 1998, Granchi et al. 1999). RFLP patterns of killer strains obtained by restriction enzymes, *Hae*III, *Msp*I and *Rsa*I, were compared with RFLP patterns of the type yeast strains from our database. The RFLP database contained restriction patterns of 54 yeast type strains that have been isolated frequently during the winemaking process according to Barnett et al. (1990). Figure 1 represents the rDNA-*Msp*I restriction patterns of killer isolates and the RFLP of appropriate type strains from the database. We were able to match 20 RFLP patterns of killer isolates with the RFLP patterns in the database. Based on that, we identified strains belonging to *S. cerevisiae, P. anomala, P. kluyveri, H. uvarum*. Identification results obtained from molecular database were confirmed by 7–12 selected biochemical and physiological tests, suggested for confirmation of individual species by Barnett et al. (1996). Results of biochemical and physiological tests (not shown) confirmed the accuracy of molecular identification. However, there were two exceptions where the RFLPs obtained did not match. To identify the isolates $S_I$1/8 and D12/2, it was necessary to perform the biochemical and physiological tests required for identification according to Barnett et al. (1990). The killer isolate D12/2 was identified as *P. pijperi* and, for this species, we have not determined any type strain. The RFLP patterns of the isolate

$S_I1/8$ and *C. rugosa* CBS $613^T$ did not match with each other, although the isolate $S_I1/8$ was identified as *C. rugosa* by the biochemical–physiological testing.

Among the 116 yeast strains isolated from a smear taken from the equipment in winery S, isolates SM1, SM2 and SM3 possessed killer activity (Table 2). All three strains were identi-

**Table 2.** Species identity and characteristics of isolated killer strains; presence (+) or absence (−) of L and M dsRNA; the strength of killer activity at different pH (+ + + strong, + + medium, + weak, − not active); sensitivity (+) and resistance (−) of killer isolates in interaction assay with killer strains type K1 and K2 at pH 3.

| Isolate | Identity and deposition number | | dsRNA | | Activity at pH | | | Interreaction | |
|---|---|---|---|---|---|---|---|---|---|
| | | | L | M | 3 | 4 | 5 | K1 | K2 |
| $S_S7/11$ | *S. cerevisiae* | ZIM 1640 | + | + | + + + | +++ | ++ | + | − |
| $S_S7/16$ | *S. cerevisiae* | ZIM 1645 | + | + | ++ | ++ | + | − | − |
| $S_S12/10$ | *S. cerevisiae* | ZIM 1667 | + | + | +++ | +++ | +++ | − | − |
| $S_S12/11$ | *S. cerevisiae* | ZIM 1668 | + | + | − | + | − | − | − |
| $S_I7/9$ | *S. cerevisiae* | ZIM 1709 | + | + | +++ | +++ | ++ | + | − |
| D8/5 | *S. cerevisiae* | ZIM 1325 | − | − | − | + | − | − | − |
| D10/4 | *S. cerevisiae* | ZIM 1349 | − | − | − | + | + | − | − |
| G12/6 | *S. cerevisiae* | ZIM 1614 | + | − | − | + | − | + | + |
| K8/4 | *S. cerevisiae* | ZIM 1423 | − | − | − | ++ | ++ | + | − |
| SM1 | *S. cerevisiae* | ZIM 1506 | + | + | +++ | +++ | ++ | + | − |
| SM2 | *S. cerevisiae* | ZIM 1510 | − | +++ | +++ | ++ | + | + | |
| SM3 | *S. cerevisiae* | ZIM 1260 | + | + | +++ | +++ | ++ | + | − |
| M1/7b | *P. anomala* | ZIM 1520 | − | − | + | ++ | − | + | + |
| M1/9 | *P. anomala* | ZIM 1521 | − | − | + | ++ | − | + | + |
| M1/10 | *P. anomala* | ZIM 1522 | − | − | + | ++ | − | + | + |
| M1/11 | *P. anomala* | ZIM 1523 | − | − | − | + | − | + | + |
| M1/12 | *P. anomala* | ZIM 1524 | − | − | + | ++ | − | + | + |
| D1/5 | *P. kluyveri* | ZIM 1314 | − | − | − | ++ | + | + | + |
| D12/1 | *P. kluyveri* | ZIM 1367 | − | − | − | ++ | + | − | − |
| D12/2 | *P. pijperi* | ZIM 1368 | − | − | − | + | − | + | + |
| $S_I1/3$ | *H. uvarum* | ZIM 1676 | + | + | − | + | − | − | − |
| $S_I1/8$ | *C. rugosa* | ZIM 1681 | − | − | − | + | − | + | + |

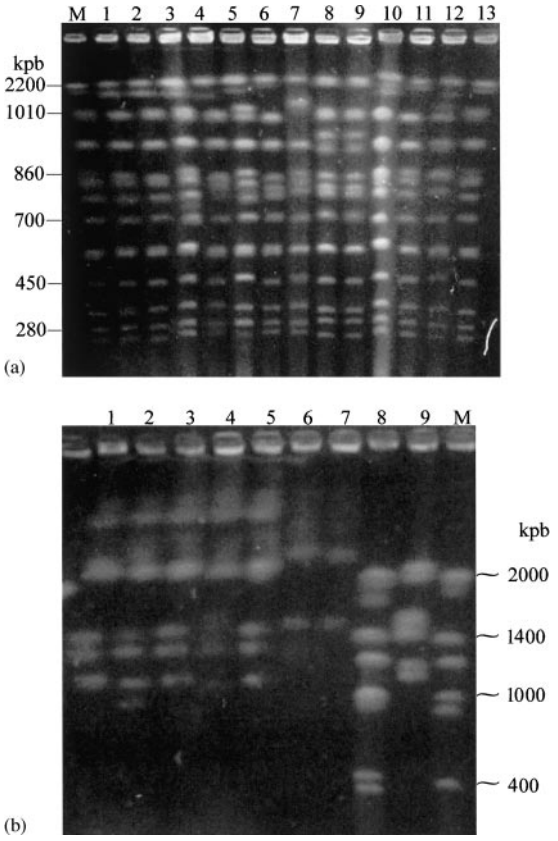


**Figure 2.** Karyotype patterns of killer isolates (a) **M.** marker; *S. cerevisiae* strains: **1.** $S_S$7/11; **2.** $S_S$7/16; **3.** $S_S$12/10; **4.** $S_S$12/11; **5.** D8/5; **6.** D10/4; **7.** SK1; **8.** SK2; **9.** SK3; **10.** $S_I$7/9; **11.** K8/4; **12.** G12/6; **13.** marker. (b) **1–5** *P. anomala* M1/7b, M1/9, M1/10, M1/11, M1/12; **6.-7.** *P. kluyveri* D1/5, D12/1; **8.** *H. uvarum* $S_I$1/3; **9.** *C. rugosa* $S_I$1/8; **M.** marker.

fied as *S. cerevisiae*. Strains SM1 and SM3 possessed stable and strong killer activity that was coded by dsRNA molecules. Strain SM2 did not contain dsRNA, however it also had a strong and stable killer character. Based on interactions of killer isolates and the K1/K2 toxin-producing strains, it can be concluded that strains SM1 and SM3 belonged to killer type K2, since killer strains of *S. cerevisiae* belonging to the same type do not interact with each other.

Killer activity of isolated strains was analysed several times during their storage. This showed that all the non-*Saccharomyces* and some *Saccharomyces* strains rapidly lost their killer activity, although all isolates indicated very strong killer activity at the time of isolation. Table 2 represents the results of killer activity determined after 2 years of storage (in glycerol at $-20^{\circ}$C), when four strains were selected for starters in small-scale fermentations. The interaction test of killer isolates and killer type K1 and K2 strains was performed at pH 3 (the pH of the must). All *P. anomala* strains were sensitive to both toxin types, as well as the strain *P. kluyveri* D1/5, isolated at the early stages of fermentation. Strain *P. kluyveri* D12/1, isolated at the end of fermentation, was resistant to both toxins, whereas *P. pijperi* D12/2 was sensitive to both of them. Strain *H. uvarum* was resistant, while the strain *C. rugosa* was sensitive to both toxin types. Strains of *S. cerevisiae* reacted differently to killer toxins K1 and K2. Several were sensitive to K1 (Table 2), while two strains (G12/6 and SM2) were sensitive to both toxins. The result indicated that the isolated killer strains *S. cerevisiae* belonged to K2 or some other type of killer toxin, different to K1 type.

*S. cerevisiae* killer strains exhibited very heterogeneous electrophoretic patterns (Fig. 2(a)). They differ in the size of large chromosomes (2200–1010 kbp) as well in the size of middle chromosomes (860–700 kbp). Karyotypes of five *P. anomala* strains belonged to three different groups. *P. anomala* isolates M1/7b, M1/10 and M1/12 exhibited identical karyotypes, whereas the karyotypes of *C. rugosa, H. uvarum* and *P. kluyveri* had specific patterns for their genera (Fig. 2(b)).

### *Fermentation characteristics of four selected killer yeasts in fermentation of Malvasia must*

Nine small-scale wine fermentations of Malvasia must were monitored. Chemical and sensory properties of four wines produced by local killer strains, possessing stable killer activity, were compared to the wines produced by commercial starter cultures and by spontaneous fermentations. All 1919 isolates were tested for their killer activity, whereas only 150 strains from the end phase of spontaneous fermentation and by local strain-induced fermentation were characterized by karyotyping.

All strains from four consecutive sampling points of inoculated fermentations (local and commercial starter cultures) showed very strong killer activity, indicating that they were able to suppress the original yeast population of Malvasia must effectively. Genetic identification allowed us to follow the succession of inoculated strain. Presented in Fig. 3(a, b) respectively, are examples of strains in the last phase of $S_S7/16$-induced fermentation and the strains in the last phase of the spontaneous fermentation. Isolates from the end-stage of inoculated fermentation (Fig. 3(a), lines 1–15) possessed identical karyotype patterns to the pattern of inoculated strain $S_S7/16$ (Fig. 3(a), line M) confirming that inoculated strain completely dominated over the indigenous yeast population of Malvasia must.

Strains isolated from spontaneous fermentation showed significant chromosomal polymorphism (Fig. 3(b)), they differed mostly in the size of larger chromosomes, between 2200 and 860 kbp. An extensive study of succession of different strains *S. cerevisiae* in five independent spontaneous fermentations of Malvasia was the object of a parallel study (Povhe Jemec et al. 2001), which showed a considerable heterogeneity of *S. cerevisiae* strains during the process of wine fermentation.

Recent work comparing the effects of different starter cultures and indigenous yeasts has shown that there are significant differences in the chemical composition of the resulting wines (Egli et al. 1999). Our results do not totally support these observations. Strains differed mostly in their ability to ferment sugars of the must. The results (Table 3) showed that the strains $S_I7/16$ and $S_S12/10$ possessed good fermentation character, since the amount of residual sugar was reduced by 98%, whereas in the spontaneous fermentation, and in the fermentations inoculated with strains $S_S7/11$ and $S_S7/16$, the sugar content was only reduced by about 90%. Commercial starters possessed an excellent ability to ferment all available sugars. All the wines produced were rich in ethanol, although some differences were observed, which were due to the strains' sugar fermentation ability. The amount of glycerol synthesized during fermentation was higher in the wines obtained by commercial starters ($7{\cdot}3\,g\,l^{-1}$) than in spontaneous or in the fermentations induced with local strains, where it reached concentrations $5{\cdot}9\,g\,l^{-1}$ and $6{\cdot}1$–$6{\cdot}8\,g\,l^{-1}$, respectively.

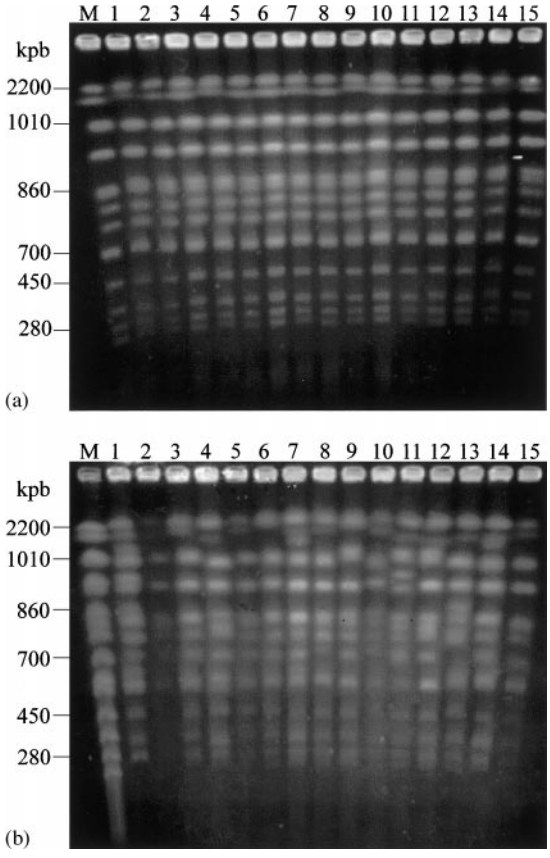


**Figure 3.** Karyotype patterns of yeast isolates from Malvasia fermentation. (a) **M.** inoculated strain $S_S7/16$; **1–15** isolated strains at the end of induced fermentation. (b) **M.** marker; **1–15** isolated strains at the end of spontaneous fermentation.

The initial amounts of free and total $SO_2$, naturally present in Malvasia must were $4{\cdot}4\,g\,l^{-1}$ and $23\,g\,l^{-1}$, respectively. Different concentrations of the final $SO_2$ reflected the strain's specific metabolism of sulfur compounds, since there was no sulfur added to the must at the beginning of fermentation. The organic acid content was slightly higher in wines obtained with indigenous starters compared to those obtained with commercial starters, whereas it was lower in spontaneous fermentation. Volatile compounds of all wines showed the most variable pattern. Strain

**Table 3.** Concentration of metabolic compounds in wines obtained in spontaneous alcoholic fermentation and with the application of commercial and indigenous yeast starters

| | Must | Wines | | | | | |
|---|---|---|---|---|---|---|---|
| | | Spontaneous | Commercial | Indigenous starters | | | |
| Compound | | A0 | X* ± SD | $S_S$7/11 | $S_S$7/16 | $S_I$7/9 | $S_S$12/10 |
| Ethanol (vol %) | n.d. | 12·7 | 13·7 ± 0·12 | 12·1 | 11·9 | 13·6 | 13·5 |
| Acetaldehyde (mg l$^{-1}$) | n.d. | 18·2 | 24·2 ± 7·84 | 24·5 | 17·9 | 37·1 | 23·6 |
| Ethylacetate (mg l$^{-1}$) | n.d. | 31·5 | 32·3 ± 4·14 | 39·9 | 27·8 | 28·7 | 35·8 |
| Methanol (mg l$^{-1}$) | n.d. | 50 | 55 ± 5·77 | 55·0 | 50·0 | 50·0 | 60·0 |
| n-Propanol (mg l$^{-1}$) | n.d. | 16·7 | 27·4 ± 3·04 | 19·6 | 22·3 | 19·7 | 19·4 |
| i-Butanol (mg l$^{-1}$) | n.d. | 31·2 | 33·0 ± 9·52 | 54·3 | 54·8 | 31·2 | 78·6 |
| i-Amylalcohol (mg l$^{-1}$) | n.d. | 195 | 236 ± 28·6 | 270 | 284 | 216 | 376 |
| Total $SO_2$ (mg l$^{-1}$) | 23 | 15·4 | 12·3 ± 2·3 | 12·5 | 14·6 | 14·6 | 11·2 |
| Free $SO_2$ (mg l$^{-1}$) | 4·4 | 4·8 | 4·3 ± 0·6 | 3·9 | 3·7 | 3·6 | 3·0 |
| Glucose (g l$^{-1}$) | 113 | 1·3 | 0 | 2·0 | 1·0 | 0·0 | 0·0 |
| Fructose (g l$^{-1}$) | 114 | 20·3 | 0 | 24·6 | 22·7 | 4·6 | 6·1 |
| Titr. Acids (g l$^{-1}$) | 9·1 | 8·7 | 9·2 | 10·6 | 10·7 | 10·4 | 9·9 |
| Citric acid (g l$^{-1}$) | 0·2 | 0·56 | 0·65 ± 0·08 | 0·9 | 0·8 | 0·9 | 1·0 |
| Tartaric acid (g l$^{-1}$) | 5·0 | 3·4 | 3·3 ± 0·16 | 3·5 | 3·5 | 3·3 | 3·6 |
| Malic acid (g l$^{-1}$) | 4·0 | 3·4 | 3·6 ± 0·20 | 4·2 | 4·2 | 4·2 | 5·0 |
| Lactic acid (g l$^{-1}$) | 0 | 0·18 | 0·092 ± 0·06 | 0·16 | 0·15 | 0·15 | 0·18 |
| Acetic acid (g l$^{-1}$) | 0 | 0·41 | 0·51 ± 0·22 | 0·62 | 0·54 | 0·49 | 0·47 |
| Glycerol (g l$^{-1}$) | 0 | 5·9 | 7·3 ± 0·7<br>6·1 | 6·1 | 6·8 | 6·1 | |
| Sensory test | | | | | | | |
| Place | | 8 | 3–6 | 7 | 9 | 2 | 1 |
| Points | n.d. | 16·81 | 17·15–16·92 | 16·82 | 16·80 | 17·20 | 17·29 |

*X ± SD: the mean value of a compound in wines obtained with 4 commercial starters with its standard deviation.
**n.d. not determined.

$S_S$12/10 produced 376 mg l$^{-1}$ i-amylalcohol and 78·6 mg l$^{-1}$ I-butanol, the largest amounts found, which may have had a beneficial impact on the wine. Acetaldehyde produced during the wine fermentation is known to be strain-specific, however, the concentrations in the fermentations of Malvasia did not vary significantly (17·9–37·1 mg l$^{-1}$) although strains of different origin were used. On the contrary, Pérez-Coello et al. (1999) observed significant differences (18·3–670·9 mg l$^{-1}$) among strains tested in fermentations of Airén variety must.

Sensory tests of wines also did not show significant differences since the maximal and the minimal points of the wines differed by only 0·49 points. Wine produced by strain $S_S$12/10 achieved 17·29 points and first place in the ranking, whereas the wine produced by strain $S_S$7/16 16·80 points and the last place. The result is in accordance with the lower content of higher alcohols in the case of strain $S_S$7/16. According to our experience while conducting this experiment, simply modifying the parameters of the individual fermentation process could produce better wines. This is already the object of another study.

## Conclusions

The strain *S. cerevisiae* is responsible for alcoholic fermentation of must into wine, but yeasts of other genera are also important in the early phase of fermentation (Yap et al. 2000). It was interesting that non-*Sacharomyces* killer strains were identified at the late fermentation stage of red wines. Five different non-*Sacharomyces* species were isolated at different phases of fermentation, which could be due to specific

red wine technology that allows longer exposure to air and equipment.

As several authors suggested, it might be of benefit to use selected indigenous yeast strains, which are supposed to have an optimal fermentation performance since they are adapted to the must in each area. Since we had a pool of killer yeasts belonging to *S. cerevisiae* all isolated from the same wine-producing region, we tried to examine this hypothesis. Other authors have performed numerous exclusion tests to select a *S. cerevisiae* with the best enological properties (Pérez-Coello et al. 1999). Our approach was straightforward, since we used indigenous killer wine yeast isolates directly as a starter culture in fermentation of the white wine Malvasia. In accordance with our first fermentation trials, we can conclude that the fermentation characteristics of indigenous killer yeast strain $S_S12/10$ were as good as those of commercial starters and is appropriate for further use as a starter culture.

Winemakers have to consider the question of whether to use starter cultures or not. Many of them do prefer to use starter cultures, since this gives them a sense of 'security'. The question as to whether indigenous yeast starters have a greater positive impact on the final product than commercial starters is still open to debate and the system 'starter culture: must/wine' has to be considered separately for every individual system.

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