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Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must

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Abstract

Fermentation by naturally occurring yeasts may produce wines of complex oenological properties that are unique to a specific region. The present work analyses the population dynamics of the yeasts during spontaneous fermentation of six varieties of grape must from the "Valle del Andarax" area (Spain). In this study we identified members of the genera *Candida*, *Hanseniaspora*, *Issatchenkia*, *Metschnikowia*, *Pichia* and *Saccharomyces* by PCR-RFLP of the ITS region. The capability of these yeasts to ferment grape must of the Macabeo variety was studied, and the volatile profile of the wine from each microvinification has been determined. Of all the yeasts isolated *Candida stellata* and *Saccharomyces cerevisiae* were able to consume virtually all the initial glucose, producing ethanol contents typical of table wines. The best profile of higher alcohols was given by *Saccharomyces cerevisiae* followed by *Hanseniaspora uvarum*, *Issatchenkia orientalis* and *Candida stellata*. *Metschnikowia pulcherrima* and *Pichia fermentans* showed the highest production of ethyl caprilate and 2-phenyl ethanol, compounds associated with pleasant aromas.

Keywords: Wine; Fermentation; Yeast identification; Volatile compounds

1. Introduction

Spontaneous population dynamics associated with a wine producing region provide information on what might be considered as the natural method to obtain a wine with specific characteristics. Selected yeasts from a wine growing area have been shown to be better adapted to specific environmental conditions and substrates (Esteve-Zarzoso et al., 2000). In spontaneous fermentation the first yeasts to appear are the apiculate yeasts (*Kloeckera*, *Hanseniaspora*) and *Candida*, followed by several species of *Metschnikowia*, *Pichia* and occasionally *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, whose characteristics are useful to preserve the oenological characteristics of wine-producing regions (Pretorious et al., 1999). The low ethanol tolerance of these yeasts and their inability to ferment all sugars present in musts are the reasons for their natural replacement by *Saccharomyces cerevisiae*. This is the main agent in alcoholic fermentation, but the natural distribution of this yeast is not clear. Some authors inform that fermentative species of *Saccharomyces* (*S. cerevisiae*) occur at extremely low populations and are rarely isolated from intact berries, suggesting that in spontaneously fermenting grapes this yeast comes from the surfaces in the winery (Martini, 1993). It has also been noted that the presence/absence of *S. cerevisiae* differs according to each plant and grape cluster (Pretorius et al., 1999). For this reason it is not always possible to obtain the same product from spontaneous wine fermentation. This problem is being solved at present by the use of commercial strains in the fermentation process, to the detriment of the wine's autochthonous character.

In this study we have isolated and studied the indigenous yeasts present in a very well-defined area. The natural habitat (Ribera del Andarax) is a valley at

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an altitude of 1.000 m limited to the north and west by mountains of over 3.000 m (Sierra Nevada), to the east by desert and to the south by the Mediterranean Sea. These geographical characteristics have made this zone an isolated ecosystem for grape production with several climatic influences: low rainfall, many hours of sunlight, and humidity and cold air from the perpetual snow. All these characteristics suggest that the natural evolution of the grapevine and the associated yeasts may have occurred at the same time. For this reason the oenological properties of the wines might be better if natural yeasts rather than commercial ones are used for fermentation.

Molecular biology methods have been applied to the identification of yeasts. DNA-based methods have the advantage of being independent of gene expression (Ness et al., 1993). In this sense, methods based on polymerase chain reaction (PCR) have been shown to be the most appropriate tools for rapid yeast identification. The differences in the rRNA region including internal transcribed spacer (ITS) and the 5.8S have been used to identify yeast species during spontaneous fermentation of natural juices such as grape (Granchi et al., 1999; Torija et al., 2001) or orange (Las Heras-Vázquez et al., 2003).

The aim of this work was to analyse potential wine yeasts isolated in the Ribera del Andarax in order to study population diversity and wine-type distribution. To elude the problems associated with the recovery of certain strains we have isolated all the yeasts possible, under the conditions of this study, present in this area. We have also analysed their influence on Macabeo (Viura) grape must by studying volatile production, sugar assimilation and other characteristics that enable yeast to impact the oenological properties of a wine.

2. Materials and methods

2.1. Yeast isolation

Yeast isolation was carried out in the Andarax valley, a wine producing area in south-east Spain. All samples were taken in September in order to ascertain which yeasts are present at harvesting. The grape varieties used for isolation were: Macabeo, Vermentino and Viognier for white wine and Merlot, Tempranillo and Cabernet Sauvignon for red wine. Of each variety five grape clusters of approximately 100 g each were harvested at random in the vineyard using totally aseptic techniques. These were then crushed aseptically in the lab and maintained together with their must, peel and seeds for 10 days at 18°C to allow spontaneous fermentation of the must and proliferation of the indigenous yeasts. Serial decimal dilutions were made in triplicate every day in a Ringer (Merck) solution, plated on YPD agar (5% yeast extract, 5% peptone, 10% glucose, 20% agar) and incubated for 2 days at 28°C. To avoid bacterial growth 100 μg ml^{-1} of ampicillin was added to the media. From these plates 15 colonies were randomly isolated from each variety of grape. The micro-organisms were separated, morphologically differentiated and classified. Four phases were considered to study the succession of micro-organism populations in each grape variety: phase I, initial population; phase II, tumultuous fermentation when 20% of the sugar is fermented; phase III, late fermentation when 70% of the sugar is fermented; and phase IV the end of fermentation when 90% of the sugar is fermented (Povhe Jemec et al., 2001).

2.2. Microbial-type cultures

Certified yeast strains of various species were obtained for controls from the Spanish Type Culture Collection (CECT) and American Type Culture Collection (ATCC). The following strains were studied: *Candida stellata* (CECT 11969); *Hanseniaspora uvarum* (CECT 10885); *Issatchenkia orientalis* (ATCC 34135); *Issatchenkia terricola* (CECT 11970); *Metschnikowia pulcherrima* (CECT 11968); *Pichia fermentans* (CECT 11773); *Saccharomyces cerevisiae* (CECT 1971).

2.3. Molecular identification of isolated strains

DNA isolation was carried out from pure cultures of each isolate as previously described (Las Heras-Vázquez et al., 2003). PCR amplification of the 5.8S-ITS region was performed in a total volume of 50 μl. Each reaction mixture contained 5–25 ng template DNA, 10 mM Tris-HCl pH 9.0, 50 mM ClK, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 μM of each primer; and 1 U *Taq* DNA polymerase (Amersham Pharmacia Biotech, Sweden). Primers ITS1 (5′-TCCGTAGGT-GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTA-TTGATATGC-3′) described by White et al. (1990), were used to amplify this region. Amplification was performed in a Perkin-Elmer Thermocycler 2400 programmed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C for denaturing, 30 s at 57°C for annealing, 1 min at 72°C for synthesis, and a final extension step of 5 min at 72°C. Amplified samples were kept at –20°C until further use. Aliquots of 10 μl of amplified product were separated electrophoretically in 1.4% (w/v) agarose gels in TBE (Tris-borate 0.045 M, EDTA 0.001 M, pH 8) buffer at 100 V constant voltage for 1 h, stained with ethidium bromide and photographed under transilluminated UV light (Fotodyne Ltd.).

For restriction analysis of the ITS region, aliquots (10 μl) of PCR products were digested without further purification with 1 U of restriction enzyme in 20 μl reaction volumes, according to the manufacturer's

instructions and conditions. The restriction enzymes used were *Cfo*I, *Hae*III and *Hinf*I. RFLP products were analysed by horizontal electrophoresis in 3% (w/v) agarose gels. DNA molecular-weight-marker (1 kbyte plus DNA ladder; Life Technologies S.A., Barcelona, Spain) was used as standard. All electrophoreses were carried out with $15 \times 7\,cm^2$ gels on a Wide Mini-Sub Cell GT unit ($15 \times 7\,cm^2$ tray with 20 wells; Bio-Rad Laboratories S.A., Madrid, Spain) at 70 V for 1 h. After electrophoresis, the gels were stained with ethidium bromide and photographed under transilluminated UV light.

Sequencing reactions were executed for both strands using ITS1 and ITS4 primers. Each sequence was repeated at least in duplicate using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems). Sequenced ITS1-5.8S-ITS2 regions were aligned using the CLUSTALW program (Jeanmougin et al., 1998).

2.4. Microvinification

The Macabeo grape variety was used for microvinification studies with all the yeasts isolated. The grape must was clarified by bentonite sedimentation $0.4\,g\,l^{-1}$ for 2 days at 4°C, autoclaved at 70°C for 10 min, and no sulphur was added before fermentation. The must was inoculated with a final concentration of 10^6 cell ml^{-1} of pure yeast culture in 250 ml bottles (Duran) in duplicate. Fermentations were carried out at 18 ± 1°C for 20 days and the bottles were covered with a sterile double gauze to avoid contamination (Mingorance-Cazorla et al., 2003). Finally, fermented products were centrifuged and stored at 4°C until analysed. Glucose, and colour were analysed using the official European Community (1990) analysis methods. Foam production was observed qualitatively comparing all the vinifications and noted as − (no foam production), and +, ++ or +++ (according to the increase in foam). Glycerol was determined using specific enzymatic kits (Boheringer-Mannheim, Germany; degree of precision of measurement ± 2%).

2.5. Gas chromatography analysis

The volatile compounds and alcohols were analysed by direct injection of 1 µl of each final product into a Varian 3900 gas chromatograph with a flame ionization detector (220°C) and a Carbowax (Supelco) column ($50 \times 0.25\,mm^2$). Both injector and detector were operated at 220°C. The carrier gases were H_2 (99.999%) at a flow rate of $35\,ml\,min^{-1}$ and N_2 (99.999%) at $36\,ml\,min^{-1}$. Injection was split mode 30 with air flow $300\,ml\,min^{-1}$. The column temperature 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.

The standard solution consisted of: methanol, acetaldehyde, ethyl acetate, 1-propanol, 2-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-butanol, capronaldehyde, isopentyl acetate, *n*-butanol, 4-methyl-2-pentenol, ethyl caproate, 2-hexanol, hexyl acetate, 1-hexanol, 2,3 butanediol, 2-phenylethanol, acetoin and ethanol, all supplied by Aldrich Chemical Company. Samples were made in triplicate and analysed statistically by Microsoft Excel 97.

3. Results and discussion

3.1. Yeast isolation and identification from spontaneous fermentation

In order to ascertain the natural yeast flora present in the area under study almost 1000 yeast isolates, 165 for each grape variety, were taken at random at different stages the spontaneous fermentation. These yeast isolates were identified according to ITS polymorphisms. The ITS1 and ITS4 primers amplified the region between 18S rRNA and 28S rRNA. The isolates showed different PCR product sizes, ranging from 375 to 880 bp (Table 1). The PCR products digested with *Cfo*I, *Hae*III and *Hinf*I enzymes were analysed for all the isolates, whereby seven different profiles were obtained in the conditions of this particular study. Five of the seven patterns were identified after comparing the molecular mass of the restriction products with those previously described (Esteve-Zarzoso et al., 1999). These five groups were *C. stellata*, *Issatchenkia terricola*, *M. pulcherrima*, *P. fermentans* and *S. cerevisiae*. A sixth profile showed the same pattern as that described for *H. uvarum* and *Hanseniaspora guilliermondii* in the above-mentioned paper. However, after an additional restriction analysis with *Dde*I endonuclease, it was identified as *H. uvarum*. In order to find the identity of the seventh profile and reconfirm the identity of the six profiles previously identified by restriction analysis of the ITS region, the PCR products from the seven groups were sequenced and compared to the available DNA sequence databases. The seventh profile showed very high sequence homology to *I. orientalis*. As a final confirmation of results, PCR-RFLP of the ITS region was applied to the seven certified species from the Spanish Type Culture Collection (CECT) and American Type Culture Collection (ATCC), obtaining total agreement in the amplification sizes and restriction profiles. When all the yeasts were identified, the seven species-specific ITS region DNA sequences were submitted to the GenBank database (Table 1).

3.2. Yeast population diversity during spontaneous fermentation

Grape must including the broken grape fruits and cluster remains was aseptically prepared in the laboratory for spontaneous fermentation. The aim of this

strategy was to avoid any contamination with the so-called "winery yeast flora" (Rosini, 1984), and to isolate only indigenous yeast microflora. Non-*Saccharomyces* yeasts were predominant in the must (phase I) and during the tumultuous fermentation (phase II) in both white and red fermentation, while *Saccharomyces* yeast was isolated in the late and end fermentation (phases III and IV) and only in Tempranillo grapes (Tables 2 and 3). In the first steps of spontaneous wine fermentation non-*Saccharomyces* species are favoured both quantitatively and qualitatively (Povhe Jemec et al., 2001; Pramateftaki et al., 2000; Torija et al., 2001; Constantí et al., 1998). Furthermore, these yeasts have been associated with the desired complexity of the wine and contribute to its flavour and quality (Romano et al., 1997). These yeasts give the wine a local character because they are adapted to specific fermentation and wine-making conditions (Pramateftaki et al., 2000).

Hanseniaspora uvarum was the main species present in must for red wine in both phases I and II, but it disappeared in phases III and IV due to its low ethanol tolerance (Table 2). *P. fermentans* was detected in Merlot variety in phase III together with *C. stellata*, which carried out the complete fermentation of this variety and Cabernet-Sauvignon. In Tempranillo variety *I. orientalis* shared fermentation phase III with

Table 1
Isolated strains, GenBank database accession number and identification according to the lengths (in pb) of the 5.8S-ITS region amplified by PCR and of the fragments obtained after digestion with three restriction endonucleases

Species	GenBank accession no.	PCR product	Restriction fragments		
			*Cfo*I	*Hae*III	*Hinf*I
Candida stellata	AY235805	475	215 + 110 + 90 + 60	475	235 + 235
Hanseniaspora uvarum	AY235806	750	320 + 310 + 105	750	350 + 200 + 180
Issatchenkia orientalis	AY235807	525	200 + 180 + 70 + 60	380 + 90 + 40	220 + 150 + 140
Issatchenkia terricola	AY235808	425	120 + 95 + 75 + 70 + 60	290 + 120	225 + 100 + 95
Metschnikowia pulcherrima	AY235809	375	205 + 90 + 80	280 + 100	190 + 175
Pichia fermentans	AY235810	450	170 + 100 + 100 + 80	340 + 90	250 + 200
Saccharomyces cerevisiae	AY235811	880	385 + 365	320 + 230 + 180 + 130	360 + 350 + 180

Table 2
Distribution of yeast (%) during spontaneous must fermentation according to fermentation phase (I–IV) and grape variety for red wine: Cabernet-Sauvignon, Merlot, Tempranillo

Fermentation phases	Red wine											
	Cabernet-Sauvignon				Merlot				Tempranillo			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Candida stellata	—	16	100	100	—	12	67	100	—	—	—	—
Hanseniaspora uvarum	100	84	—	—	100	88	—	—	100	100	2	—
Issatchenkia orientalis	—	—	—	—	—	—	—	—	—	—	67	2
Issatchenkia terricola	—	—	—	—	—	—	—	—	—	—	—	—
Metschnikowia pulcherrima	—	—	—	—	—	—	—	—	—	—	—	—
Pichia fermentans	—	—	—	—	—	—	33	—	—	—	—	—
Saccharomyces cerevisiae	—	—	—	—	—	—	—	—	—	—	32	98

Table 3
Distribution of yeast (%) during spontaneous must fermentation according to fermentation phase (I–IV) and grape variety for white wine: Macabeo, Vermentino, Viognier

Fermentation phases	White wine											
	Macabeo				Vermentino				Viognier			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Candida stellata	—	—	—	—	—	—	18	100	—	—	82	100
Hanseniaspora uvarum	100	89	—	—	—	33	52	—	100	42	—	—
Issatchenkia orientalis	—	—	—	—	—	—	—	—	—	—	—	—
Issatchenkia terricola	—	11	100	100	100	67	30	—	—	4	—	—
Metschnikowia pulcherrima	—	—	—	—	—	—	—	—	—	54	18	—
Pichia fermentans	—	—	—	—	—	—	—	—	—	—	—	—
Saccharomyces cerevisiae	—	—	—	—	—	—	—	—	—	—	—	—

S. cerevisiae, which was also present in phase IV. In the white wine varieties (Table 3) *H. uvarum* was present in phases I and II of Macabeo and Viognier fermentation. In the Vermentino variety, however, it was detected in phases II and III, but not in phase I. *M. pulcherrima* was present in phases II and III of the Viognier must fermentation, which was completed by *C. stellata*. Much of the Macabeo fermentation was carried out by *I. terricola*, but this strain was overtaken by *C. stellata* in the Vermentino variety.

In Macabeo spontaneous fermentation only two different yeasts were obtained and neither of which gave rise to an optimal alcohol level (*H. uvarum* 3.81% and *I. terricola* 7.97%, Table 4). For Vermentino, Viognier, Cabernet-Sauvignon, Merlot and Tempranillo fermentation phases I and II were characterized by the growth of ethanol-sensitive yeasts (*I. terricola*, *H. uvarum*, *M. pulcherrima*, *P. fermentans* and *I. orientalis*) and finished by a yeast able to produce 13–14% ethanol (Table 4), such as *C. stellata* and *S. cerevisiae* in phases III and IV. Fermentative species of *Saccharomyces* occur at extremely low populations in the vineyard environment, less than 10 colony forming units per square cm of fruit surface or gram of soil (Martini et al., 1996). In our study the percentage present in phases III and IV in Tempranillo grapes represented 63 of the 960 yeasts isolated in total. *Saccharomyces* is the predominant fermenting species colonizing the surfaces of the winery, up to 80% of the whole winery resident yeast flora (Martini, 1993), and as our results confirm, it is rarely isolated from intact grapes or aseptically pressed must. *C. stellata* has proved to be the main species present in must from the Priorat region (Torija et al., 2001), the major micro-organism in the fermentation of sherry (Esteve-Zarzoso et al., 2001) and the Carinyena grape variety (Constantí et al., 1998) both in Spain, as well as in Trebbiano grapes (Romano et al., 1997), and in the Sangiovese variety (Tuscany) (Granchi et al., 1999) in Italy. Our results are in accordance with others reporting that the presence of *Candida* is associated with juices with high sugar concentrations such as grape must (Mora et al., 1990).

3.3. Production of volatile compounds after must microvinification by each single-isolated strain

The capacity of all these isolated yeasts to ferment grape must is of interest in the wine-making process. The Spanish Macabeo variety produces a light, acid, floral and fairly fruity white wine, but the aroma and flavour soon dissipate so it should be drunk quite young. The aim of this study was to investigate the impact of naturally occurring yeast isolated on wine characteristics. White wine is fermented without grape skins, but yeasts present on them are incorporated into the must in the crushing process. The first step was to check certain oenological properties of the isolated yeasts. Table 4 shows the fermentation criteria investigated in the study. The criteria related to fermentation vigor were ethanol and glycerol production, and glucose remaining after fermentation. Other criteria observed were foam production and optical density at 420 nm as an indicator of the yellow intensity and transparency index.

Only *C. stellata* and *S. cerevisiae* were able to consume virtually all initial glucose (199.08 gr l^{-1}) to produce ethanol contents typical of table wines. *C. stellata* is described as the predominant yeast in fermenting musts in some regions (Ciani and Maccarelli, 1998; Torija et al., 2001), probably due to its high capacity for ethanol production. Glycerol is a by-product of fermentation that has a slightly sweet taste leaving an impression of smoothness on the palate. In no case did glycerol production exceed 5.2 g l^{-1}, which is the threshold taste level of sweetness (Noble and Bursick, 1984). The highest level was presented by *P. fermentans* and *H. uvarum*, which produce low amounts of ethanol.

The colour of a wine is an indicator of its condition, quality, age, and even style. Generally speaking, the less intense the colour, the more delicate the flavour and body will be, and the colour of any good wine should be clear. The least intense colour was produced by *S. cerevisiae* and the most intense by *I. orientalis*, and foam formation by the latter is another handicap for its use. It is clear that the *S. cerevisiae* isolated in this work

Table 4
Results of the microvinification of Macabeo grape must by individual yeasts

Strain	Ethanol production[a]	Glucose remaining[b]	Glycerol production[c]	Foam formation	Colour[d]
Candida stellata	13.48±0.17	0.69±0.07	0.16±0.00	++	0.06±0.00
Hanseniaspora uvarum	3.81±0.29	92.23±0.13	2.31±0.03	–	0.09±0.01
Issatchenkia orientalis	2.52±0.54	101.95±14.30	0.11±0.00	+++	0.12±0.00
Issatchenkia terricola	7.97±0.17	33.98±2.84	0.03±0.00	+	0.09±0.00
Metschnikowia pulcherrima	5.37±0.32	43.69±5.27	1.64±0.03	–	0.09±0.02
Pichia fermentans	5.98±0.53	38.88±4.18	2.31±0.05	+	0.09±0.00
Saccharomyces cerevisiae	14.45±0.35	0.25±0.03	0.57±0.01	–	0.04±0.01

[a] % (v/v).
[b] Residual glucose gr l^{-1}.
[c] g l^{-1}.
[d] O.D. at 420 nm.

is well adapted to wine fermentation, as it assimilates sugar rapidly. Indigenous yeasts present in a specific wine producing region may yield wines of unique character due to the production of various combinations of volatile compounds.

The fermentations of Macabeo must (Table 5) with all the isolated yeasts gave different volatile profiles. Of these, *I. terricola*, as well as showing low fermentative characteristics, must be discarded for mixed fermentation due to its high ethyl acetate production (222.11 mg l^{-1}), above the limit of 150 mg l^{-1} which produces a vinegary taste (Rapp and Mandery, 1986).

Average values of acetaldehyde range from 40 mg l^{-1} for red wine and about 80 mg l^{-1} for white wine, to 300 mg l^{-1} for sherries (Liu and Pilone, 2000). *H. uvarum* showed the highest value (259.07 mg l^{-1}) and *I. orientalis* the lowest (72.60 mg l^{-1}), so all yeasts were within the above-mentioned range values and may be used in wine fermentation. The term higher alcohols refers to those possessing more than two carbon atoms with a higher molecular weight and boiling point than ethanol. They have a significant influence on the taste and character of wine (Lambrechts and Pretorius, 2000). With regard to the total of 2-butanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol, the best profile was given by *S. cerevisiae* (237.97 mg l^{-1}) followed by *H. uvarum* (201.27 mg l^{-1}), *I. orientalis* (170.60 mg l^{-1}) and *C. stellata* (164.46 mg l^{-1}). *M. pulcherrima* and *P. fermentans* showed the lowest values. However, these two strains showed the highest production of ethyl caprilate, 2-pheyl ethanol and a very good level of 2,3-butanediol. Esters of higher alcohols are associated with pleasant aromas, and in particular ethyl caprilate is associated with a pear aroma (Lambrechts and Pretorius, 2000). The concentration of 2,3-butanediol has been used as to differentiate the character of wine as it is a normal constituent and one of the most abundant (Romano et al., 1998). It may have an effect on the wine bouquet, and its viscosity contributes favourably to the wine body. Table 5 shows that in order to improve the *S. cerevisiae* 2,3-butanediol production we can use a mixture of this yeast with *M. pulcherrima* or *P. fermentans*. Finally, 2-phenyl ethanol is characterized by a delicate fragrance of rose petals, and it is the main commercial alcohol after ethyl alcohol (Huang et al., 2001). *M. pulcherrima* and *P. fermentans* together with *S. cerevisiae* showed the best profile of 2-phenyl ethanol production.

Based on ethanol production and glucose consumption (Table 4), only *C. stellata* and *S. cerevisiae* could be used as fermentative strains in pure culture. However, *C. stellata* gives a product with less glycerol content than *S. cerevisiae*, produces more foam and accumulates ethyl acetate and acetoin.

Other authors have proposed the mixture of yeasts and the sequential addition of cultures (Herraiz et al.,

Table 5
Concentration and comparative study of major volatile compounds obtained by microvinification with the 7 isolated strains. Fermentation was carried out in duplicate at 18°C for 20 days and each sample was injected in triplicate

	Acetaldehyde	Ethyl acetate	Methanol	2-Butanol	2-Methyl-1-propanol	2-Methyl-1-butanol	3-Methyl-1-butanol	Acetoin	Ethyl caprilate	2,3-Butanediol	2-Phenyl ethanol
Candida stellata	188.00±5.98	74.89±4.02	33.86±2.57	42.45±0.26	10.19±2.28	36.07±2.58	75.75±3.01	42.34±3.69	0	16.25±2.91	35.29±3.98
Hanseniaspora uvarum	259.07±9.07	69.65±3.53	61.29±0.11	40.40±1.27	4.11±0.40	42.69±0.05	114.47±6.44	18.98±0.62	0	121.76±6.90	47.47±2.07
Issatchenkia orientalis	72.60±4.37	0	58.99±1.45	0	18.03±0.92	34.12±0.95	118.51±3.17	0	0	0	53.77±2.87
Issatchenkia terricola	183.34±4.21	222.11±0.03	43.54±2.12	27.48±0.13	17.47±0.11	71.44±4.39	139.37±6.21	6.06±0.37	0	68.08±0.33	120.55±6.45
Metschnikowia pulcherrima	73.43±3.81	62.07±0.30	0	0	9.37±0.01	0	0	11.01±0.24	128.79±0.82	68.55±1.34	249.19±6.65
Pichia fermentans	100.63±0.27	52.19±0.10	35.54±4.95	0	3.78±0.35	0	0	0	227.03±7.40	42.21±0.02	151.72±5.88
Saccharomyces cerevisiae	93.80±0.02	0	35.54±4.95	31.16±0.67	7.85±0.71	61.69±2.74	137.27±7.22	0	0	60.91±1.53	189.90±8.46

The values are, therefore, the mean of six injections and are expressed in mg l^{-1}.

1990; Zironi et al., 1993). Tables 4 and 5 give the criteria to choose the appropriate yeast to be used at each stage of fermentation. Thus, to emulate natural fermentation it is advisable to start with a low fermentation yeast that appears in the initial phase (phase I) such as *H. uvarum*, followed by a typical yeast of tumultuous or late fermentation (phases II or III) such as *I. orientalis*, *M. pulcherrima* or *P. fermentans*, and finishing with a yeast that consumes all the remaining sugar, such as *S. cerevisiae*.

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