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Contribution of different natural yeasts to the aroma of two alcoholic beverages

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Abbreviations: CECT – Colección Española de Cultivos Tipo; GM – grape must; OJ – orange juice; SC – sugar cane

Summary

The aroma formation in the fermentation of two types of natural musts by 12 different yeasts has been analysed. In grape must fermentation *Pichia fermentans* Colección Española de Cultivos Tipo (CECT) 11773, *Clavispora lusitaniae* OJ6 and *Pichia anomala* OJ5 produced the best balance between concentrations of ethyl acetate and high alcohols. When orange juice was fermented with the 12 yeasts, *Pichia fermentans* CECT 11773, *Rhodotorula mucilaginosa* OJ2 and *Hanseniaspora uvarum* CECT 10885 produced a good beverage with low alcoholic grade. For both types of natural musts *Pichia fermentans* CECT 11773 increased the presence of higher alcohols and ethyl acetate. After using this strain both alcoholic beverages obtained the highest evaluation in the sensory analysis.

Introduction

When referring to wine aroma it is important to distinguish between the aroma from the grapes (collection and processing), the aroma produced by fermentation and the bouquet produced by the transformation of the aroma during ageing. The majority of volatile compounds of which grape aroma is composed are known to be constituents of many other fruits. There are compounds that precipitate with the must slurry, such as aromatic hydrocarbons, aliphatic *n*-alkanes and *n*-alkenes. In certain varieties, only a few esters contribute to the aroma and these are acetate esters of short chain alcohols. Thus, when the grapes are pressed, C_6-aldehydes and alcohols are formed enzymatically. However, these compounds have also been identified in the presence of enzyme inhibitors. It is, therefore, very difficult to determine to what extent these compounds are formed by the grape varieties. Other compounds, such as monoterpenes, sesquiterpenes and ketones are present in some varieties of grape (Rapp & Mandery 1986).

The wine aroma appears mainly during yeast fermentation. Ethanol is the main component followed by diols, higher alcohols and esters. Ethanol determines the viscosity (body) of wine and acts as a fixer of aroma. Although the quantity of organic acids in the wine is small, they are sufficiently volatile to contribute to its aroma. The most important of these organic acids are acetic acid, propanoic acid, butanoic acid and lactic acid, and the latter three are usually below the perception threshold. Esters are present in small amounts in grapes, but their formation is parallel to ethanol formation. The yeast used in the fermentation process has a great influence on ester production. The basic aroma of wine has been attributed to four esters (ethyl acetate, isoamyl acetate, ethyl caproate and caprylate), two alcohols (isobutyl and isoamyl) and acetaldehyde, while the remaining compounds only modify the basic aroma (Avakyants *et al.* 1981).

Finally, the bouquet of a wine depends on the storage conditions. It may be due to the presence of aldehydes and acetals from the oxidation process, or to the reduction which takes place after ageing, although the latter is of less importance. Red wine benefits from the aromatic elements extracted from the oak barrels, such as phenolic compounds or diastereomers. Apart from this, oxygen penetrates through the wood and changes the flavours. During storage excess acetates in young white wines gradually hydrolyse, becoming acids and alcohols. The decrease in these acetates could be responsible for the loss of freshness and fruitiness. All of these compounds are produced by the yeasts in concentrations which are very close to the equilibrium concentrations of ester, fatty acid and ethanol. Other changes in the ageing of white wine are: a decrease in monoterpene alcohols (linalool, geraniol and citrenol), an

increase in oxides, and the formation of other monoterpenes (Rapp & Mandery 1986).

In the same way as the positive flavour compounds in wine-making come from three sources (grapes, fermentation and ageing), the undesirable odours are classified as those derived from the cultivar, fermentation and processing. The most important of these is hydrogen sulphide, which smells of rotten egg and is produced during fermentation. Another undesirable odour is the vinegary smell produced by acetic acid and ethyl acetate. When bacterial proliferation occurs lactic odour appears, due to malolactic fermentation. The equilibrium of these compounds is important in a fermented must in order to determine whether they are able to produce the desired flavour compound and not the undesirable odours (Suárez-Lepe 1997).

The spontaneous fermentation involved in the first period of aroma production is carried out by the so-called non-*Saccharomyces* strains, such as *Kloeckera*, *Hanseniaspora*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Hansenula* and *Metschnikowia*. These strains have been described as producers of high concentrations of some compounds whose influence on the sensory quality of wine has been reported (Rojas *et al*. 2001). These spontaneous yeasts are developed during the first 4–6 days, after which they die due to their inability to tolerate ethanol concentrations of over 6% (Gil *et al*. 1996). Non-*Saccharomyces* yeasts can impart desirable or undesirable flavour-determinants to the fermented must. *Hanseniaspora* and *Pichia* are able to promote the esterification of various alcohols such as ethanol, geraniol, isoamyl alcohol and 2-phenylethanol, thus increasing concentrations of esters with a fruity aroma (Rojas *et al*. 2001). These yeasts are, therefore, most useful for the production of volatile compounds. Consequently, their isolation, the type of aromas they are able to produce, their resistance to ethanol and the type of must they are able to ferment are all of great interest.

Bearing in mind that the present trend in the wine industry is to develop new, original products, mixed or sequential cultures of non-*Saccharomyces* or authoctonous yeasts together with *S. cerevisiae* may be a good method of to conferring a particular aroma and characteristics to wines and other alcoholic beverages (Zironi *et al*. 1993). For this reason, two isolation processes were carried out to find yeasts from natural sources which are able to ferment natural juices. By studying the aroma formation produced in the fermentation of two natural musts by 12 different yeasts, this work aims to select a yeast which produces the most adequate aromatic profile.

Materials and methods

Microbial strains

All yeasts used in this study are listed in Table 1. Two are commercial (CM) *Saccharomyces* strains used in the wine industry (*Saccharomyces cerevisiae* L2056, *Saccharomyces cerevisiae* Colección Española de Cultivos Tipo (CECT) 1351). Seven were isolated from orange juice (OJ) and selected for their high capacity to produce volatiles (Las Heras-Vázquez *et al*. 2003) (*Pichia fermentans* CECT 11773, *Rhodotorula mucilaginosa* OJ2, *Trichosporum asahii* OJ3, *Pichia anomala* OJ4, *Hanseniaspora uvarum* CECT 10885, *Saccharomyces cerevisiae* OJ5, *Clavispora lusitaniae* OJ6). One was isolated from sugar cane (SC) (*Pichia anomala* SC1), while the last two were isolated from grape must (*Hanseniaspora uvarum* GM1, *Metschnikowia zobellii* GM2).

The isolation from OJ was carried out by mixing juice and peel of oranges from the Andarax valley in Almería (Spain) in sterile conditions. To avoid bacterial growth 100 μg ml^{-1} of ampicillin was added to the mixture. The juice was maintained at 18 °C for 20 days to allow the proliferation of native yeast. Aliquots were taken every

Table 1. Source and evolution of yeast strains used in this study.

Yeast	Sensory analysis		
	Source	Grape must	Orange juice
Clavispora lusitaniae OJ6	OJ	4	3
Hanseniaspora uvarum CECT 10885	OJ	1	4
Hanseniaspora uvarum GM1	GM	2	2
Metschnikowia zobellii GM2	GM	1	2
Pichia anomala OJ4	OJ	3	1
Pichia anomala SC1	SC	2	2
Pichia fermentans CECT 11773	OJ	5	5
Rhodotorula mucilaginosa OJ2	OJ	3	4
Saccharomyces cerevisiae CECT 1351	CM	3	1
Saccharomyces cerevisiae L2056	CM	5	2
Saccharomyces cerevisiae OJ5	OJ	2	1
Trichosporum asahii OJ3	OJ	2	1

GM – grape must, OJ – orange juice, SC – sugar cane, CM – commercial strains. Sensorial study of the product formed by fermentation of GM and OJ with 12 strains of yeast.

day, diluted to 10^{-5} or 10^{-6} in Ringer solution and spread in YPD media. The plates were incubated at 29 °C and colonies appeared in 2 days. Each of the selected colonies was separated and analysed. Microorganisms were first differentiated morphologically and separated. They were then classified by PCR-RFLP of the ITS region and sequencing. Microorganisms from GM were isolated and classified in the same way after being collected aseptically and crushed in a sterile jar. The must obtained was fermented at 18 °C for 20 days with 100 μg of ampicillin ml^{-1} and aliquots were taken each day. The samples for GM were taken from the Alpujarra area in Almería (Spain). The isolation of *Pichia anomala* SC1 was carried out taking aliquots from each stage of industrial alcohol production (Montero Inc. Motril) from SC diluted to 10^{-5} or 10^{-6} in Ringer solution and spread in YPD media. The isolated colonies were separated and analysed.

Microvinification

The GM was treated with 0.4 g l^{-1} of bentonite for 2 days at 4 °C before fermentation and the OJ was centrifuged at 5000 × *g* for 10 min at 4 °C. Both were then inoculated with 10^6 cell of each yeast strain ml^{-1} and fermentations were carried out at 18 ± 1 °C for 20 days. In both cases the bottles were covered with a sterile double gauze layer to avoid contamination. Fermentation was carried out in laboratory conditions using 12 pure yeast cultures. The fermented products were centrifuged and stored at 4 °C until analysed.

Sensory analysis

The fermented products were analysed sensorially and chemically. The sensorial study was evaluated on a scale from 1 to 5, where one represented low intensity of flavour and five a very high intensity. This evaluation was carried out by two professional wine tasters. Different fermented products were presented in random order at 10 °C in standard wine-tasting glasses, covered with a watch-glass to avoid the escape of components (ISO, 1977) (Ubeda-Iranzo *et al*. 2000). The results of this evaluation were only used to support the analytical results.

Gas chromatography analysis

Direct injection of the samples was not possible due to the residual sugar in the fermented products. Fermented OJ or GM was distilled from a volume of 100 to 25 ml. From the distillate 1 μl was injected into a Varian 3900 gas chromatograph with a flame ionization detector (220 °C) and a Carbowax (Supelco) column (50 × 0.25mm). Both injector and detector were operated at 220 °C. The carrier gas was H_2 (99.999%) at a flow rate of 35 ml min^{-1}. The column temperature was programmed from 40 to 120 °C at a rise rate of 7 °C min^{-1} and 4 min at 120 °C.

The standard solution was distilled and the recovery factor of each compound was determined. Methanol: standard concentration 3979.90 mg l^{-1}, recovery factor 3.75, acetaldehyde: standard concentration 3959.79 mg l^{-1}, recovery factor 2.41, ethyl acetate: standard concentration 4522.61 mg l^{-1}, recovery factor 3.53, 1-propanol: standard concentration 4040.20 mg l^{-1}, recovery factor 1.61, 2-methyl-1-propanol: standard concentration 4070.35 mg l^{-1}, recovery factor 2.37, 3-methyl-1-butanol: standard concentration 4070.35 mg l^{-1}, recovery factor 4.01, 2-methyl-1-butanol: standard concentration 4115.58 mg l^{-1}, recovery factor 3.63 were supplied by Montero Inc., ethanol: standard concentration 1% (v/v), recovery factor 1.32 and acetoin: standard concentration 1000 mg l^{-1}, recovery factor 3.92 by Aldrich Chemical Company. Samples were analysed in triplicate, and the mean result was divided by the concentration factor (4) and multiplied by the recovery factor of each compound.

Results and discussion

From the GM isolation, 148 yeasts were analysed, but only *Hanseniaspora uvarum* GM1 and *Metschnikowia zobellii* GM2 were different. These strains were isolated and classified by PCR-RFLP of the ITS region. Seven strains from OJ isolated in our laboratory were also used for the fermentation (Las Heras-Vázquez *et al*. 2003). Finally we have included in the present work one strain isolated from SC (Table 1).

From the sensory analysis several strains could be discarded due to their poor taste and aroma (Table 1). *Hanseniaspora uvarum* GM1, *Mestchnikowia zobellii* GM2 from GM, *Trichosporum asahii* OJ3, *Hanseniaspora uvarum* CECT 10885, *Rhodotorula mucilaginosa* OJ2 from OJ and the SC isolate (*Pichia anomala* SC1) gave the worst results when GM was fermented. Moreover, these yeasts were not able to consume all the sugar (Table 2). *Saccharomyces cerevisiae* CECT 1357 and *Saccharomyces cerevisiae* OJ5 were able to consume all the sugar, but the sensory analysis did not produce very good results owing to their low capacity to produce higher alcohols (Table 2). The sommelier described the product as having a tasteless, 'bakery-like' aroma.

Small amounts of higher alcohols contribute positively to wine quality, while excessive amounts may detract from quality. Higher alcohols are important as precursors for ester formation during ageing (Gil *et al*. 1996). We have studied the potential of all the strains to ferment GM and OJ and produce the following volatile compounds which are involved in the wine flavour: ethanol, acetaldehyde, ethyl acetate, methanol, *n*-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol (Table 2). None of them produced methanol (data not shown).

Several authors have shown the vital role of yeasts in the formation of aroma (Suomalainen 1971). To

Table 2. Concentration and comparative study of major volatile compounds present in GM and wine fermented in the wine-cellar by *Saccharomyces cerevisiae* L2056, and products obtained by microvinification with 12 yeast strains.

	Acetaldehyde	Ethyl acetate	Ethanol[a]	1-propanol	2-methyl-1-propanol	2-methyl-1-butanol	3-methyl-1-butanol	Acetoin	pH	Glucose[b]
GM non-fermented	80.16 ± 2.71	0	0.11 ± 0.01	0	0	0	0	0	3.27	25.85
Wine produced in wine-cellar	431.61 ± 8.12	50.19 ± 1.76	14.03 ± 0.62	4.05 ± 0.64	3.79 ± 0.07	20.02 ± 0.29	109.32 ± 0.91	60.05 ± 2.47	3.65	0
Clavispora lusitaniae OJ6	724.37 ± 2.46	26.33 ± 1.27	2.23 ± 0.10	1.16 ± 0.09	3.36 ± 0.15	8.98 ± 0.23	27.87 ± 0.24	200.43 ± 9.90	3.53	13.83
Hanseniaspora uvarum CECT10885	71.84 ± 4.29	0	0.04 ± 0.00	0	0	0	0	106.09 ± 22.71	3.28	25.65
Hanseniaspora uvarum GM1	90.06 ± 3.35	0	0.10 ± 0.01	0	0	0	0	214.05 ± 9.50	3.33	27.99
Metschnikowia zobellii GM2	110.16 ± 3.27	0	0.08 ± 0.01	0	0	0	0	0	3.29	25.70
Pichia anomala OJ4	1311.33 ± 18.32	9.67± 1.12	17.12 ± 0.20	10.57 ± 0.25	8.91 ± 0.25	97.09 ± 0.37	152.06 ± 0.54	67.47 ± 4.82	3.55	0.03
Pichia anomala SC1	0	109.89 ± 2.56	0.32 ± 0.05	0	0	0	0	51.51 ± 10.11	3.72	24.53
Pichia fermentans CECT 11773	132.21 ± 2.17	164.18 ± 1.54	2.94 ± 0.01	1.94 ± 0.11	2.16 ± 0.06	20.72 ± 0.31	59.31 ± 0.16	226.25 ± 7.71	3.27	21.30
Rhodotorula mucilaginosa OJ2	160.67 ± 5.39	0	0.05 ± 0.00	0	0	0	0	0	3.60	24.99
Saccharomyces cerevisiae CECT 1357	0	0	15.71 ± 0.15	0	0	0	0	0	3.74	0
Saccharomyces cerevisiae L 2056	344.09 ± 11.61	55.63 ± 1.96	14.35 ± 0.21	5.84 ± 0.27	2.84 ± 0.06	25.03 ± 0.98	103.34 ± 0.42	97.35 ± 7.75	3.58	0
Saccharomyces cerevisiae OJ5	0	10.27 ± 0.54	3.27 ± 0.14	0	0	0	20.29 ± 0.16	73.98 ± 2.57	3.48	27.81
Trichosporum asahii OJ3	0	0	0.08 ± 0.00	0	0	0	0	47.79 ± 2.14	3.41	25.35

Fermentation was carried out at 18 °C for 20 days. Fermentation was in triplicate and, after distilling, each sample was injected in triplicate. The values are, therefore, the mean of nine injections and are expressed in mg l^{-1}.

[a] % (v/v).

[b] residual sugars: g glucose/100 ml.

determine this influence we compared the gas chromatography (GC) profiles of the must fermented with the 12 yeasts in the laboratory with that of CM wine made from the same Macabeo grape variety (Table 2). The CM wine was produced with *Saccharomyces cerevisiae* L2056 in the wine cellar, and we repeated the fermentation in our laboratory. The GC profile was quite similar except for acetaldehyde, whose increase in CM wine may be due to the oxidation of ethanol during the ageing process in the bottle. Moreover, in laboratory fermentation not all the glucose was consumed. Very similar aromatic products have been obtained after fermentation with *Pichia fermentans* CECT 11773, *Clavispora lusitaniae* OJ6 and *Pichia anomala* OJ5. This is in accordance with the sensorial study, where *Pichia fermentans* CECT 11773 scored 5 *Clavispora lusitaniae* OJ6 scored 4 and *Pichia anomala* OJ5 scored 3. The lower scores for *Clavispora lusitaniae* OJ6 and *Pichia anomala* OJ4 may be due to the abnormally high acetaldehyde concentration (724.4 and 1311.3 mg l^{-1} respectively), which has an important effect on the sensory results. Furthermore, the high level of isoamyl alcohol and isobutanol may have a negative impact on the organoleptic quality of the wine (Sipiczki *et al.* 2001). Thus, *Pichia fermentans* CECT 11773 showed the best profile to ferment GM, and although it has a low capacity to produce ethanol, it does have a high capacity to produce volatile compounds which increase the aromatic properties of wine in a mixed culture.

Ethyl acetate has a pleasant aroma which turns vinegary above 150 mg l^{-1}. Between 50 and 150 mg l^{-1} the ester contributes to the hard character of the wine (Rapp & Mandery 1986). High concentrations of ethyl acetate do not improve the aroma of young wines, but these negative effects are reduced during bottle ageing. For this reason *Pichia fermentans* CECT 11773 ethyl acetate concentration does not have a negative influence, and it can be used to improve wine aroma (Lilly *et al.* 2000; Rojas *et al.* 2001).

The sensory and chemical analyses indicate that the best products correspond to the samples with a greater concentration of higher alcohols, acetates and ethyl esters, which contribute to the aroma quality in young wine. This is in accordance with the study developed by Lema *et al.* (1996), where the contribution to the aroma quality by non-*Saccharomyces* yeasts was shown. The ratio between the contents of ester and higher alcohols is described as a good measure of the contribution of a given yeast to the aroma of wine (Mateo *et al.* 1991). Table 3 shows the rate obtained for *Saccharomyces cerevisiae* L2056, *Pichia fermentans* CECT 11773, *Pichia anomala* OJ4, and *Clavispora lusitaniae* OJ6. *Pichia fermentans* and *Clavispora lusitaniae* showed the highest values. There is no information about the production of volatile compounds by fermentation with *Pichia fermentans* and *Clavispora lusitaniae*, because they do not appear naturally in GM. However, this study suggests that they are able to produce a good product with low alcoholic grade. *Pichia anomala* OJ4 has been shown to be a good producer of ethanol and higher alcohols, but not of ethyl acetate. Nonetheless, Rojas *et al.* (2001) have shown that *Pichia anomala* 10590 has high capacity to esterify alcohols and suggested its possible use in mixed starters for wine production (Rojas *et al.* 2001).

Several authors have described must fermentations by non-*Saccharomyces* yeasts: *Hanseniaspora guillermondii*, *Kloeckera apiculata* (Romano *et al.* 1992, 1997a; Zironi *et al.* 1993; Gil *et al.* 1996), *Pichia anomala* (Rojas *et al.* 2001), *Candida stellata*, *Torulaspora delbrueckii* (Ciani & Maccarelli 1998), *Candida valida*, *Brettanomyces bruxellensis*, *Rhodotorula aurantica*, *Dekkera intermedia* (Mateo *et al.* 1991; Romano *et al.* 1997b), *Candida cantarellii* (Toro & Vázquez 2001), all from GM. However, we have developed an organoleptically equilibrated product by the fermentation of GM with *Pichia fermentans*, *Pichia anomala* and *Clavispora lusitaniae* yeasts originally present in other sources such as OJ (Las Heras-Vázquez *et al.* 2003; Arias *et al.* 2002), lager breweries (van der Aa Kühle & Jespersen 1998) or bakeries (Arlorio *et al.* 1999).

When OJ was used to produce an alcoholic beverage, a low production of higher alcohols was detected (Table 4). This low concentration of higher alcohols may be due to an insufficient amino acid concentration in OJ to produce the same quantity as in the GM fermentation (Zoecklein *et al.* 2001). The ideal quantity of these compounds is between 140 and 210 mg l^{-1}, and the quantity of products formed from OJ is lower in all cases (Table 5). In the sensory analysis *Pichia fermentans* CECT 11773, *Rhodotorula mucilaginosa* OJ2 and *Hanseniaspora uvarum* CECT 10885 produced a very good taste product. None of the yeasts were able to consume all the OJ sugar, increasing the instability of the final product. Only *Saccharomyces cerevisiae* L2056 was able to metabolize all the sugar and produce the highest alcohol grade (Table 4), but the taste was rotten, obtaining a score of only two in the sensory analysis (Table 1). The presence of methanol might be expected

Table 3. Production of ethyl acetate, higher alcohols and the ratio between the ester and alcohol contents (E/A).

	Ethyl Acetate (mg l^{-1})	Higher alcohols (mg l^{-1})	E/A
Wine produced in brewery	50.19	137.18	0.37
Clavispora lusitaniae OJ6	26.33	41.37	0.64
Pichia anomala OJ4	9.67	268.63	0.04
Pichia fermentans CECT 11773	163.18	84.13	1.95
Saccharomyces cerevisiae L2056	55.63	136.99	0.41

Table 4. Concentration and comparative study of major volatile compounds present in OJ and in the products obtained by microvinification with 12 yeast strains using OJ.

	Acetaldehyde	Ethyl acetate	Methanol	Ethanol[a]	1-propanol	2-methyl-1-propanol	2-methyl-1-butanol	3-methyl-1-butanol	Acetoin	pH	Glucose[b]
Orange juice non-fermented	53.19 ± 1.92	0	183.03 ± 19.90	0.74 ± 0.03	0	0	0	0	0	3.74	6.32
Clavispora lusitaniae OJ6	0	0	73.83 ± 1.37	1.68 ± 0.05	0	1.89 ± 0.04	0	12.75 ± 0.4	457.40 ± 42.28	3.81	4.79
Hanseniaspora uvarum CECT 10885	0	49.28 ± 0.56	99.25 ± 0.65	3.92 ± 0.06	0	0	0	0	0	3.84	0.54
Hanseniaspora uvarum GM1	0	16.06 ± 0.40	69.97 ± 0.24	3.01 ±0.01	0	0	0	6.29 ± 0.28	415.53 ± 17.46	3.88	1.87
Metschnikowia zobellii GM2	0	0	35.52 ± 0.20	1.51 ± 0.07	0	0	0	5.37 ± 0.32	142.18 ± 6.60	3.73	5.73
Pichia anomala OJ4	0	12.60 ± 0.25	50.91 ± 0.96	1.45 ±0.01	0	0	0	9.91 ± 0.50	76.33 ± 5.62	3.65	4.99
Pichia anomala SC1	0	60.86 ± 0.18	22.35 ± 0.28	0.11 ± 0.01	0	0	0	0	379.39 ± 8.37	3.92	6.00
Pichia fermentans CECT 11773	0	13.87 ± 0.25	22.53 ± 0.20	3.75 ± 0.10	0	1.13 ± 0.08	0	0	192.45 ± 10.25	3.81	1.76
Rhodotorula mucilaginosa OJ2	0	13.09 ± 0.78	67.12 ± 0.86	1.29 ±0.02	0	0	0	9.55 ± 0.36	415.35 ± 17.75	3.81	4.12
Saccharomyces cerevisiae CECT 1357	53.09 ± 0.27	7.34 ± 0.18	27.38 ± 0.55	1.03 ± 0.03	0	1.52 ± 0.09	0	0	352.98 ± 5.98	3.62	5.72
Saccharomyces cerevisiae L 2056	43.55 ± 1.45	0	43.45 ± 0.40	2.94 ±0.06	0	0	0	16.92 ± 0.45	256.59 ± 15.85	4.00	1.96
Saccharomyces cerevisiae OJ5	58.85 ± 1.21	0	88.14 ± 1.59	5.27 ±0.08	2.064 ± 0.08	0	18.5 ± 0.61	62.92 ± 1.26	266.11 ± 8.05	3.81	0
Trichosporum asahii OJ3	0	0	60.05 ± 0.84	1.02 ± 0.03	0	1.59 ± 0.04	0	8.98 ± 0.41	271.19 ± 2.28	3.90	5.43

Fermentation was carried out at 18 °C for 20 days. Fermentation was in triplicate and after distilling each sample was injected in triplicate. The values are, therefore, the mean of nine injections and are expressed in mg l^{-1}.

[a] % (v/v).

[b] residual sugars: g glucose/100 ml.

Table 5. Production of ethyl acetate, higher alcohols and the ratio between the ester and alcohol contents (E/A).

	Ethyl acetate (mg l^{-1})	Higher alcohols (mg l^{-1})	E/A
Clavispora lusitaniae OJ6	60.86	0	–
Hanseniaspora uvarum CECT10885	49.28	0	–
Hanseniaspora uvarum GM1	16.06	6.29	2.55
Pichia anomala OJ4	12.60	9.91	1.22
Pichia fermentans CECT 11773	13.87	1.13	12.27
Rhodotorula mucilaginosa OJ2	13.09	9.55	1.37
Saccharomyces cerevisiae CECT1357	7.34	1.52	4.83
Saccharomyces cerevisiae L2056	0	16.92	–

to be problematic in OJ fermentation, since the pectin concentration is higher in OJ than in GM (Zoecklein *et al*. 2001). However the methanol level in this new beverage is not a problem, as it is lower than that of wine, which is in the range of 100–200 mg l^{-1} (Kourkoutas *et al*. 2002).

It is interesting to determine the acetoin concentration as it is involved in wine bouquet, particularly in apiculate species (Romano & Suzzi 1996). Acetoin levels produced by *Saccharomyces cerevisiae* in GM fermentation are in accordance with those described by Romano & Suzzi (1993): from non-detectable amounts to 194.6 mg l^{-1}. Thus *Saccharomyces cerevisiae* L 2056 may be considered as having a 'high acetoin production' phenotype (97.35 mg l^{-1}) which is chosen to confer desirable flavour of the final product. This is, in fact, the strain used in the wine-cellar. Although *Saccharomyces cerevisiae* OJ5 is also a good acetoin producer, it produces a smaller amount of higher alcohols than L2056. The level of acetoin produced by other non-*Saccharomyces* strains is high, with the exception of *Metschnikowia zobellii* GM2 and *Rhodotorula mucilaginosa* OJ2. Moreover, neither of these strains are very good candidates to ferment GM, due to their low level of production of alcohol and other compounds. The low acetoin concentration in the OJ fermentation shows that the level of acetoin produced depends on the media (Romano & Suzzi 1996). It would also be interesting to investigate whether the high acetoin concentration in GM is due to induction of the enzymes involved in its production, or because acetoin is not converted and therefore accumulates.

The present study has found some yeasts that could be used to improve the body of the wine, increasing the concentration of ethyl acetate and higher alcohols. Furthermore, we have used these yeasts to develop a new product from OJ with low alcoholic grade and a very fresh taste (Mingorance-Cazorla *et al*. 1999).

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