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Influence of sequential yeast mixtures on wine fermentation

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Abstract

The use of *Pichia fermentans* in pure cultures and sequential mixtures with *Saccharomyces cerevisiae* has been studied to improve the aromatic compounds and characteristics of a wine. *P. fermentans* has proved to be a good starter strains for must fermentation in the winemaking industry. It has shown the same level of sulphur tolerance and the same growth rate as *S. cerevisiae*. We have demonstrated that only 2 days of must fermentation with *P. fermentans* in sequential mixtures are enough to increase the following compounds in the wine both qualitatively and quantitatively: acetaldehyde, ethyl acetate, 1-propanol, *n*-butanol, 1-hexanol, ethyl caprilate, 2,3-butanediol and glycerol. Maintaining this non-*Saccharomyces* strain in contact with the must for longer periods quantitatively increases the flavour composition.

Keywords: *Pichia fermentans*; *Saccharomyces cerevisiae*; Sequential mixture fermentation; Wine

1. Introduction

In the wine making process, the type and amount of aroma depend on the following: yeast, environmental factors (climate, soil), cultivar, fruit condition, vinification process, must pH, amount of sulphur dioxide, amino acids present in the must and malolactic fermentation (Lilly et al., 2000). Several non-*Saccharomyces* yeasts have been described in must fermentation: *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 and Maccarelli, 1998), *Candida valida*, *Brettanomyces bruxellensis*, *Rhodotorula aurantica*, *Deckera intermedia* (Mateo et al., 1991; Romano et al., 1997b) and *Candida cantarellii* (Toro and Vázquez, 2002). Non-*Saccharomyces* yeasts improve the wine bouquet, but are not able to complete fermentation due to their low ethanol tolerance. For this reason, several authors have studied fermentation with mixtures of yeasts, either applied simultaneously (Moreno et al., 1991; Gil et al., 1996; Erten, 2002) or in sequential cultures (Herraiz et al., 1990; Zironi et al., 1993; Toro and Vázquez, 2002).

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Yeast contributes to wine flavour in three main ways: it influences the ecology of the winemaking process, the metabolism and enzymatic activities and the organoleptic impact of individual species or combinations of species on wine flavour. The characteristic fruit flavours of wine are primarily due to a mixture of hexyl acetate, ethyl caproate, ethyl caprilate, isoamyl acetate and 2-phenylethyl acetate (Falqué et al., 2001). Some of these aroma compounds have specific functions in the yeast cell, while others are still speculative (Lambrechts and Pretorius, 2000). Higher alcohols are important precursors for the formation of esters, which are associated with pleasant aromas. They can be produced during alcoholic fermentation through the conversion of the branched chain amino acids present in the medium (valine, leucine, isoleucine, threonine and phenylalanine), or produced de novo from a sugar substrate. Their exact function is unknown, but they may serve to detoxify any aldehydes produced during amino acid catabolism or be involved in the regulation of amino acid anabolism. Moreover, oxidative deamination provides the yeast with a mechanism for obtaining nitrogen. For this reason, amino acid metabolism to higher alcohols is restricted to the exponential growth phase (Vollbrecht and Radler, 1973). The fresh and fruity aroma derives in large part from the mixture of esters.

The fruit tree arome is linked to ethyl esters, while the tropical fruit notes are linked to acetates of higher alcohols. The formation of ethyl acetate during fermentation involves two steps. Alcohols become esterified by reacting with fatty acids which have undergone a previous activation by combining with coenzyme A. Fatty acids with a chain length of C8–C14 act as anti-microbial compounds, but when esters are formed these fatty acids are eliminated. Yeast metabolism influences the final composition of the wine and this metabolism is different for each strain.

The autochthonous microflora of grapes is highly variable, with a predominance of low alcohol-tolerant strains of *Hanseniaspora*, *Kloeckera* and various species of *Candida*, whereas *S. cerevisiae* is present only in small amounts (Lema et al., 1996). During the first phases of spontaneous alcoholic fermentation, the non-*Saccharomyces* yeasts flourish, contributing significantly to wine aroma. In the following stages, a succession of different ethanol-tolerant *Saccharomyces* strains are established and this completes the synthesis of volatile fermentative compounds (Fleet, 1990; Mateo et al., 1991).

To date, must grape fermentation by *Pichia fermentans* has not been reported. In this study, we use this yeast for increasing the aromatic composition of the wine in combination with *S. cerevisiae*. When yeasts were screened for good combinations of aromatic compounds produced during grape must fermentation, *P. fermentans* CECT 1173 proved to have the best profile. This yeast has a high capacity to produce volatile compounds, which increase the aromatic properties of wine, although it has shown a low capacity to produce ethanol (Mingorance-Cazorla et al., 2003).

The current trend in the wine industry is to develop new technologies and the use of mixed or sequential cultures of non-*Saccharomyces* together with *S. cerevisiae* that can contribute to this end. These studies may help to confer a particular aroma and characteristics according to the potential of each strain. For this reason, we have studied the flavour compounds produced by fermentation, capacity to produce alcohol, sulfite resistance and the rate and capacity to ferment sugar in must using two types of yeasts: *Saccharomyces cerevisiae* L2056, *P. fermentans* CECT11773 and sequential mixtures of both.

2. Materials and methods

2.1. Microvinifications

The minimum medium (MM) consisted of: 1.34% of yeast nitrogen base without ammonium sulphate and amino acids (YNB, Difco, Detroit, USA) and 4% glucose supplied by Merck Farma Quimica (Barcelona, Spain). The grape variety employed in this study was Macabeo (Viura), a Spanish variety that produces a light, acid, floral and fairly fruity white wine whose aroma and flavour soon dissipate so it should be drunk quite young. The grape must was treated with 0.4 g l^{-1} of bentonite for 2 days at 4 °C to clarify it by sedimentation before fermentation, and inoculated with 5% of *pied du cuve*, an overnight preculture of the yeast in must. The bottles with 250 ml of MM media or grape must were covered with a sterile two-layer gauze to avoid contamination, and fermentations were carried out in duplicate at 18 °C±1 °C for 20

days, inoculating 10^6 ufc/ml of *S. cerevisiae* or *P. fermentans* for each medium.

2.2. Sequential fermentations

The mixed fermentations were carried out with aliquots of 250 ml of sterile must maintained at 18 °C using 10^6 ufc/ml of *P. fermentans* for 2, 3, 4, 6 or 8 days. After each of these time intervals, 10^6 ufc/ml of *S. cerevisiae* was added to form the mixture of yeasts, which were then maintained up to a total of 20 days in all cases. All these sequential fermentations were made in duplicate. The wines produced were centrifuged and stored at −20 °C, for a maximum of one month, to be analysed together.

2.3. GC analysis

The volatile compounds and alcohols were analysed, in triplicate, by direct injection of 1 μl of each final product into a Varian 3900 gas chromatograph with a flame ionisation detector (220 °C) and a CP-WAX 57CB column (50×0.25 mm) supplied by Varian Iberica (Madrid, Spain). Both injector and detector were operated at 220 °C. The carrier gases was N_2 (99.999%) at a flow rate of 36 ml/min. Injection was split mode 30 with air flow 300 ml/min. All the gases were supplied by Praxair España (Malaga, Spain). The column temperature was programmed from 40 °C for 2 min to 120 °C at a rise rate of 3 °C/min and 4 min at 120 °C. Samples were analysed in triplicate. The identification and quantification of compounds were carried out by comparing retention time and concentration with the standard solution (Sigma-Aldrich, Química, Barcelona, Spain) used in the alcohol industry.

2.4. SO_2 tolerance

Sulfite tolerance of both strains was measured, in four independent experiments, before fermentation. YPD medium (yeast extract 10 g/l, peptone 20 g/l and glucose 20 g/l) was prepared varying the quantities of SO_2: none, 0.1 and 0.2 g/l. Each medium was inoculated separately with 10^6 cells/ml of *S. cerevisiae* or *P. fermentans* and all of them were maintained for 48 h at 28 °C in constant agitation. Aliquots were taken each 2 h and plated in YPD medium to calculate the cfu/ml. The tolerance to SO_2 was measured as the velocity of growth, calculated as the slope of the time versus the logarithm of the colony forming units.

3. Results and discussion

Non-*Saccharomyces* yeasts contribute to the final characteristics of wine due to their capacity for producing high concentrations of certain fermentation compounds, such as glycerol, esters and acetoin (Romano et al., 1993, 1997a; Romano and Suzzi, 1993), whose influence on the sensory quality of wine has been discussed previously (Fleet et al., 1984; Mateo et al., 1991; Gil et al., 1996; Ciani and Maccarelli, 1998; Rojas et al., 2001). Research into the isolation of non-*Saccharomyces* yeasts has, therefore, been considered a priority for the winemaking industry. In recent years, yeasts have been isolated which produce large amounts of glycerol (Toro and Vázquez, 2002), acetate esters (Rojas et al., 2001) and other fermentation by-products (Ciani and Maccarelli, 1998; Zironi et al., 1993; Lema et al., 1996) for use in the winemaking industry. In a previous study, a strain of *P. fermentans* was isolated and classified by PCR-RFLP of the ITS region and sequencing (GenBank accession no. AY027508) (Las Heras-Vázquez et al., 2003). Biochemical analysis showed that it produced a very balanced combination of grape must fermentation by-products which substantially improved the organoleptic characteristics of the wine (Mingorance-Cazorla et al., 2003). This strain proved to be capable of forming up to 5.03% ethanol in grape must and was tolerant to ethanol concentrations above 6%.

European regulations on wine production limit sulphur dioxide content to a maximum concentration of 0.2 g/l. Sulphur dioxide is necessary to stabilise the biochemical and chemical properties of wine. It also inhibits oxidation and wine browning by combining irreversibly with phenolic quinones to form colourless addition products. This makes these constituents unfavourable substrates for polyphenol oxidases. Sulphur dioxide is the only effective antimicrobial agent against wine lactic acid bacteria (Romano and Suzzi, 1993), and greatly inhibits the growth of non-*Saccharomyces* yeasts, resulting in the variation of the aromatic characteristics of the fermented musts (Heard and Fleet, 1985; Herraiz et al., 1989). Elimination of

SO_2 from current winemaking techniques would imply unacceptable wine spoilage by yeast and bacteria. Consequently, it is of interest to study the tolerance of *P. fermentans* CECT 11173 for its use in the winemaking process. In sterile YPD medium with 2% glucose *P. fermentans* showed the same level of tolerance to sulphur dioxide as *S. cerevisiae* (L2056) (Fig. 1), a commercial strain for inoculation in directed fermentation processes. Both yeasts maintain their biological activity in the presence of 0.1 g/l sulphur dioxide, but their activity decreases when sulphur dioxide exceeds 0.2 g/l. This effect was more marked in *P. fermentans*, whose cells die 170 h sooner than those of *S. cerevisiae*. Growth rates (μ) in the presence of permitted levels of sulphur were significantly different according to Student's *t*-test ($t_{calculate}9.98 > t_{tabulate}2.45$, $\alpha=0.05$), but this sulphur concentration (0.1 g/l) allows the development of both cultures (Table 1). Nevertheless, this result shows that *P. fermentans* can be used as a starter strain in industrial must fermentations. When non-*Saccharomyces* yeasts such as *Kloeckera*, *Torulaspora* and *Candida* are used in wine fermentation together with *Saccharomyces*, they are rapidly eliminated by the sulphur dioxide added in the first stages of the industrial process (Herraiz et al., 1990; Toro and Vázquez, 2002).

After 20 days, fermentation by-products formed in the medium by *P. fermentans* and *S. cerevisiae* were compared (cultures I and II in Table 2). In

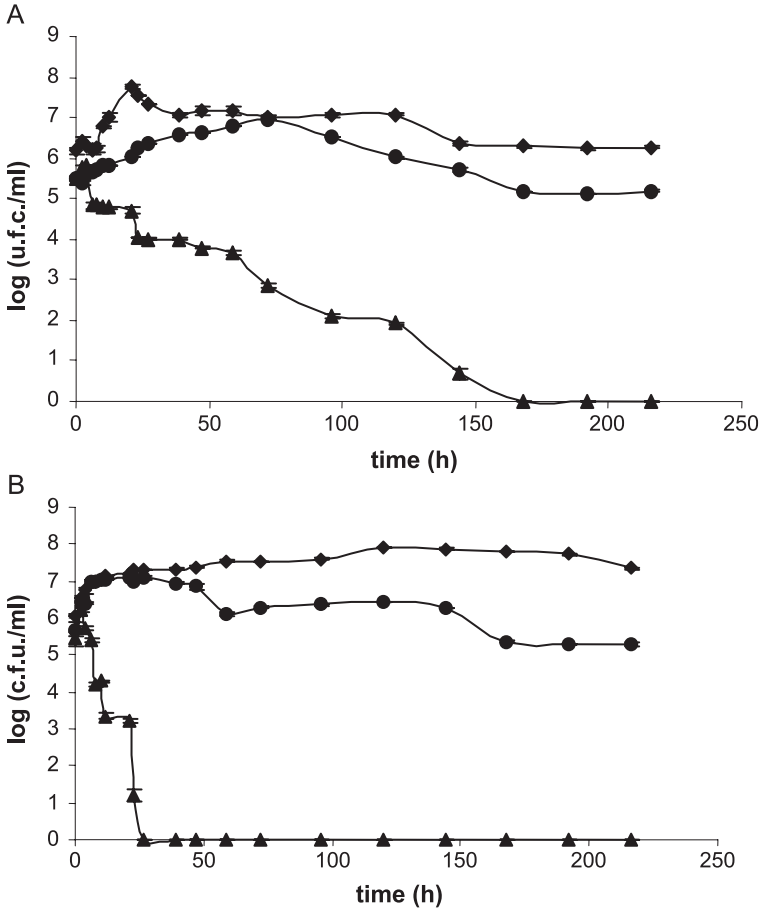

Fig. 1. Effect of SO_2 addition on the growth of *S. cerevisiae* (A) and *P. fermentans* (B). YPD medium was prepared in three conditions: non-SO_2 (♦), 0.1 g/l (●), 0.2 g/l (). The cultures were inoculated with 10^6 colony forming units (cfu)/ml and were maintained at 28 °C in constant agitation. Aliquots were taken and plated to count the number of colonies per millilitre at each time.

Table 1
Growth rate of *S. cerevisiae* and *P. fermentans*

	S. cerevisiae (L2056)		*P. fermentans* (CECT11773)		t^a (α=0.05)
	$\bar{x}$ (n=4)	S.D.	$\bar{x}$ (n=4)	S.D.	
Non-SO_2	0.059	0.008	0.059	0.018	0.075
0.1 mg/l SO_2	0.033	0.002	0.058	0.004	9.98
0.2 mg/l SO_2	−0.067	0.008	−0.186	0.015	14.21

S.D.: standard deviation.
The cultures were made in YPD medium without SO_2, with 0.1 and with 0.2 g/l. All the cultures were inoculated with 10^6 colony forming units, and aliquots were taken each 2 h during 1 day to measure the growth rate (μ). μ was calculated as the slope of the logarithm of the cfu/ml against the time in hours.
[a] Tabulate Student's t-test for $\upsilon(n_1+n_2-2)=2.45$.

minimal medium without amino acids and with 4% glucose as the only carbon source, no significant differences in growth were observed and the formation of volatile compounds was very poor. In MM medium the production of 2-methyl-1-butanol by *S. cerevisiae* was similar to the production in must ($t_{calculate}=0.092<t_{tabulate}=2.23$, $\alpha=0.05$). Likewise, 3-methyl-1-butanol production by *S. cerevisiae* was similar in both media. These compounds were not synthesised by *P. fermentans* in MM media. However, *P. fermentans* did produce these compounds in similar quantity to *Saccharomyces* in a complex medium formed by Macabeo grape must (cultures III and IV). In this medium, *P. fermentans* synthesised nearly twice as much acetaldehyde and three time less ethyl acetate. It should be pointed out that production of 2,3-butanediol was five times greater in *P. fermentans* than in *S. cerevisiae*, and that glycerol was only produced by *P. fermentans*. As expected, *Saccharomyces* produced approximately twice as much ethanol as *P. fermentans*.

In order to increase the amount of aromatic compounds synthesised by *P. fermentans*, the sequential mixed culture was optimised. Sterile Macabeo must was spread with *P. fermentans* up to 10^6 cfu/ml. It was then inoculated after 2, 3, 4, 6 or 8 days with *S. cerevisiae* up to 10^6 cfu/ml and fermented to a total of 20 days. Table 2 contains the composition of fermentation products obtained in cultures V to IX. Acetaldehyde production increased by 33% from the first days of incubation with *P. fermentans* when compared to cultures with only *S. cerevisiae*. Acetaldehyde content did not improve with an increase in the number of days of fermentation with either *P. fermentans* or the mixed culture.

Ethyl acetate content was the sum total synthesised by both yeasts, but this amount did not depend on the difference in culture time between the two yeasts. The amount of this ester was 21% greater in the mixed culture than in culture with only *S. cerevisiae*. Ethyl acetate must be present in wines at concentrations below the threshold taste level of 150 mg/l (Jackson, 1994). This limit was not exceeded by the yeasts assayed in either the preliminary or the sequential cultures, although in the latter the concentrations were higher and they remained constant irrespective of culture time with non-*Saccharomyces* yeast. The accumulation of by-products such as acetoin and ethyl acetate can have a negative effect on wine (Ciani and Maccarelli, 1998). With the fermentation procedure described in the present work, acetoin is eliminated and ethyl acetate is present well below the acceptable limits.

Methanol did not undergo changes at different incubation times, nor did it increase due to the synthesis of the two yeasts. The same behaviour was also observed for 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol. In all cases of mixed fermentation, ethanol content was approximately 10%. As *P. fermentans* cannot surpass 6% ethanol content in culture we must conclude that *S. cerevisiae* is responsible for completing the synthesis of this alcohol in all the mixed cultures. *P. fermentans* proved to produce 39% more *n*-butanol than *S. cerevisiae*. The level of this alcohol was, therefore, greater in the mixed cultures than in the conventional ones, and it increased gradually in proportion to culture time with *P. fermentans*.

In *S. cerevisiae* cultures in MM medium or grape must, 1-hexanol was not detected. However, *P. fermentans* produced 213.42 mg/l of this compound in grape must, increasing to 260.29 mg/l when fermentation was started with *P. fermentans* for 8 days and *S. cerevisiae* was added for the remaining 12 days. Once again, the presence of both yeasts gave rise to greater production of an aromatic compound. Unexpectedly, when cultures IV and V were compared, it was found that all the 1-hexanol formed by *P. fermentans* was produced in the first 2 days of fermentation, and the remainder, produced during mixed fermentation, was probably due to a metabolic interaction with *S. cerevisiae* (cultures V to IX). Gil et al. (1996) found that certain compounds, one

Table 2
Aromatic composition (in mg/l) of the wine produced with pure and mixed cultures

	Type of fermentation								
	I	II	III	IV	V	VI	VII	VIII	IX
Acetaldehyde	193.59±3.51	170.38±0.81	205.76±22.01	345.46±23.89	307.13±29.59	328.05±39.55	350.13±33.38	311.15±26.38	262.37±24.37
Ethyl acetate	0	11.87±2.72	67.23±3.68	28.92±3.24	81.61±10.35	83.44±4.49	84.96±2.78	83.16±8.45	83.43±10.54
Methanol	0	0	44.53±5.19	77.63±8.63	44.1±3.23	43.26±6.23	44.8±7.25	44.63±3.58	44.36±3.28
Ethanol[a]	4.01±0.69	2.03±0.08	10.61±1.50	5.03±0.55	10.73±1.71	9.74±1.19	8.96±0.76	8.98±0.27	10.25±0.48
1-Propanol	0	0	27.79±1.41	28.69±2.36	29.90±4.18	29.71±2.87	28.63±1.32	29.63±2.45	29.49±0.91
2-Methyl-1-propanol	0	0	69.02±1.14	69.67±2.33	69.92±1.49	69.79±2.10	69.67±1.86	69.65±2.95	69.87±2.39
n-Butanol	0	0	51.88±5.36	84.31±4.22	86.53±3.12	90.07±5.64	90.91±4.47	91.02±6.23	90.32±8.24
2-Methyl-1-butanol	120.01±8.21	0	120.19±5.67	120.89±7.34	120.94±9.81	120.31±7.87	121.08±6.50	120.80±5.78	120.50±7.62
3-Methyl-1-butanol	159.75±11.03	0	161.07±11.18	154.93±11.65	163.02±6.43	163.99±12.89	170.29±10.88	167.55±13.29	165.42±8.85
1-Hexanol	0	0	0	213.42±11.68	220.59±17.12	209.18±3.08	223.71±4.72	256.66±7.45	260.29±3.87
Ethyl caprilate	252.66±3.26	253.89±4.77	257.95±27.89	253.83±9.47	258.73±7.17	279.82±24.08	349.08±4.56	365.32±7.24	392.29±11.59
Acetic acid[b]	0.029±0.001	0.033±0.02	0.026±0.001	0.024±0.001	0.032±0.002	0.034±0.002	0.028±0.001	0.025±0.001	0.027±0.001
2,3-Butanediol	28.97±6.97	29.53±3.12	23.48±6.11	124.29±1.93	127.86±17.76	317.81±30.17	312.92±5.43	317.21±16.06	397.68±13.69
Acetoin	0	0	0	187.59±17.39	0	0	0	0	0
Glycerol	0	0	0	1440±17.30	60±1.36	390±7.31	990±15..36	1110±21.89	1690±31.25

All the fermentations were made at 18 °C with 0.1 g/l SO_2. The first two fermentations were made using minimum media (MM) (1.34% of yeast nitrogen base without ammonium sulphate and amino acids (YNB), 4% glucose) and the others were made using Macabeo grape must. I: MM fermented with *S. cerevisiae* L2056 for 20 days. II: Fermentation of MM with *P. fermentans* CECT 11773 for 20 days. III: Fermentation of Macabeo grape must for 20 days with *S. cerevisiae* L2056. IV: Fermentation of Macabeo grape must with *P. fermentans* for 20 days. V: Fermentation of grape must for 2 days with *P. fermentans* followed by addition of *S. cerevisiae* until 20 days. VI: Fermentation of grape must with *P. fermentans* for 3 days followed by additon of *S. cerevisiae* until 20 days. VII: Fermentation of grape must with *P. fermentans* for 4 days followed by addition of *S. cerevisiae* until 20 days. VIII: Fermentation of grape must with *P. fermentans* for 6 days followed by addition of *S. cerevisiae* until 20 days. IX: Fermentation of grape must with *P. fermentans* for 8 days followed by addition of *S. cerevisiae* until 20 days. Glycerol was determined using specific enzymatic kits Boheringer-Mannheim, Germany.

[a] % (v/v).

[b] g/l.

of which is 1-hexanol, are varietal compounds, characteristic of the variety used. In the present study, 1-hexanol was only observed when must was fermented with *P. fermentans*. It was not detected in unfermented must or must fermented with *Saccharomyces*, which would suggest that it was synthesised by the non-*Saccharomyces* yeast.

On the other hand, glycerol synthesis was proportional to incubation time with *P. fermentans*, up to a maximum of 1.69 mg/ml. As with 1-hexanol, glycerol production also increased in the presence of both yeasts (by 15%). Surprisingly, the opposite effect was obtained with respect to acetoin production by the interaction of the two yeasts. This compound was produced by *P. fermentans* in grape must, but was not detected in any of the mixed fermentations assayed. Acetoin production levels are comparable to those of other strains which have been described as good producers of this compound (Ciani and Maccarelli, 1998). It seems obvious that the presence of *S. cerevisiae* prevents acetoin accumulation in all media, although it is a potential acetoin source. This effect has not been described previously, and it may indicate that *Saccharomyces* makes active use of acetoin as a precursor for another compound not studied in the present work. Herraiz et al. (1990) described that *K. apiculata* has a retarding effect on the growth of *S. cerevisiae*. Levels of 2-methyl-1-butanol and 3-methyl-1-butanol seem to be correlated with fermentative ability, inasmuch as yeasts used in this work ferment very well.

The case of 2,3-butanediol is interesting, because when *P. fermentans* was used to ferment grape must its production increased by 76% with respect to the quantity obtained in MM medium (from 29.53±3.12 to 124.29±1.93 mg/l). This would suggest that the type of medium is a determining factor for 2,3-butanediol production. Mixed fermentations were even more productive, giving rise to a 68% increase from the second to eighth day of incubation with *P. fermentans*. Production of this important aroma was also greater in the presence of both yeasts. Production after 2 days in mixed culture was almost twice that of *P. fermentans* alone. This suggests a synergetic effect between these yeasts for the synthesis of 2,3-butanediol. This differs greatly from previous observations of mixed or sequential cultures with *Hanseniaspora* or *Kloeckera* and *Saccharomyces*, which showed a slight decrease in production of this compound (Zironi et al., 1993).

P. fermentans produced less acetic acid than *S. cerevisiae* in individual cultures in grape must. In all combinations assayed, levels of acetic acid did not exceed those obtained in fermentation with only *Saccharomyces*. The fact that *P. fermentans* does not originate from grape juice means that it possesses certain characteristics of potential value in enology. Thus, acetic acid content in cultures with *P. fermentans*, whether on its own or mixed with *Saccharomyces*, will be extremely low compared to those obtained by other non-*Saccharomyces* yeasts such as *Torulaspora delbruecki* or *C. stellata* (Ciani and Maccarelli, 1998).

On the whole, mixed fermentation with *P. fermentans* and *S. cerevisiae* produced a substantial increase in the presence of aromatic compounds such as acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl caprilate, 2,3-butanediol and glycerol. When compared to other non-*Saccharomyces* yeasts, *P. fermentans* does not produce large amounts of compounds involved in wine aroma. Values of glycerol, acetoin and ethyl acetate obtained by strains of *T. delbrueckii*, *C. stellata*, *Hanseniaspora uvarum* and *K. apiculata* were one or two orders of magnitude greater than those obtained by *P. fermentans* (Ciani and Maccarelli, 1998). However, with respect to glycerol formation, there is little difference between *K. apiculata* and *H. guillermondii* (Romano et al., 1997a) and the *P. fermentans* studied in the present work. On the other hand, *P. fermentans* proved to produce more acetaldehyde than the aforementioned strains.

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