Physiological features of *Schizosaccharomyces pombe* of interest in making of white wines

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Abstract This work studies the physiology of *Schizosaccharomyces pombe* strain 938 in the production of white wine with high malic acid levels as the sole fermentative yeast, as well as in mixed and sequential fermentations with *Saccharomyces cerevisiae* Cru Blanc. The induction of controlled maloalcoholic fermentation through the use of *Schizosaccharomyces* spp. is now being viewed with much interest. The acetic, malic and pyruvic acid concentrations, relative density and pH of the musts were measured over the entire fermentation period. In all fermentations in which *Schizo. pombe* 938 was involved, nearly all the malic acid was consumed and moderate acetic concentrations produced. The urea content and alcohol level of these wines were notably lower than in those made with *Sacch. cerevisiae* Cru Blanc alone. The pyruvic acid concentration was significantly higher in *Schizo. pombe* fermentations. The sensorial properties of the different final wines varied widely.

Introduction

The *Schizosaccharomcyes* genus possesses interesting metabolic properties that can be of use in white winemaking. However, *Schizosaccharomcyes* is not used due to certain off-flavours commonly associated with their metabolism. There is no doubt that they are commonly isolated from wines suffering from strong organoleptic and chemical deviations through the appearance of acetic acid, H_2S, acetaldehyde, acetoin and ethyl acetate [1–3].

On the other hand, not only because these yeasts possess high fermentative power [4, 5] but also due to their capacity to metabolise malic acid, these yeasts are now being viewed with notable interest, especially for the elaboration of wines with high acidity levels from northern Spanish wine-growing regions such as Galicia, the Basque Country, Rioja and Navarra. Under these conditions where growth cycles are short, grapes accumulate excessively high quantities of malic acid, and its elimination is particularly necessary.

Until now, the lactic acid bacteria *Oenococcus oeni* and *Lactobacillus plantarum* have been the most traditionally used organisms to remove malic acid from musts and wines especially in red winemaking [6, 7]. However, the malolactic fermentation performed by these microorganisms is one of the most complicated processes in enology due to growing requirements [8].

White young wines are usually produced without induction of malolactic fermentation in order to preserve young aroma characteristics. But under the aforementioned

climatic conditions, total or partial demalication through the induction of *Schizosaccharomyces spp.* could be highly recommendable. Maloalcoholic fermentation could preserve young aroma characteristics and, at the same, reduce the 'green apple sourness' that malic acid brings to wine when it is present in high levels. Furthermore, *Schizosaccharomcyes pombe* and *Schizosaccharomcyes malidevorans* readily grow in musts and wines, rendering the use of lactic acid bacteria unnecessary, maintaining grape variety and yeast fermentation aromas.

Different research lines have also been developed to solve problems in this regard linked to the genetic modification of fermentative *Saccharomyces* spp. by integrating *Oenococcus oeni* malolactic enzyme gene (mleA) that makes them capable of decarboxylating malate to lactate [9–12]. Regardless of the controversy over genetically modified organisms (GMO), their use at industrial level is restricted by European legislation (EC Regulation N° 479/2008) and subjected to numerous controls concerning the 'substantial equivalence' between a conventional product and a transgenic one.

Schizosaccharomyces spp. shows a stronger autolytic release of parietal polysaccharides than the one showed by *Sacch. cerevisiae,* due to their particular cell wall formation; this could be of interest in white wines aged over lees [13].

Other recent findings also suggest that *Schizosaccharomyces spp.* possesses urease activity [14] that can be used in order to reduce the urea content during fermentation processes [15]. This compound is the main precursor of ethyl carbamate (a toxic compound) in wine [16].

Taking into account the above, in wine production the presence of some non-*Saccharomyces* yeasts, such us *Schizosaccharomyces spp.*, causes considerable interest [1, 17–20]. *Schizosaccharomyces spp.* could be used and immobilised in alginate [17, 21–23] or in mixed and sequential fermentations with *Saccharomyces* spp. [20] to soften its occasional negative sensorial impacts [24]. These fermentation technologies have presented significant results in the deacidification of wine on a laboratory scale [21, 25].

The use of *Schizosaccharomyces spp.* alone, or with other yeast species in combined fermentations, could reduce wine standardisation as described by some authors [18, 26, 27], increasing the complexity and aroma profile of modern wines [28]. Contradictory results have been obtained in various studies that examined the impact of *Schizosaccharomyces spp.* on sensorial analysis and volatile compound composition [25, 29–31]. For example, this genus has been related to high levels of ethyl acetate, acetaldehyde [30] or acetic acid [15, 31]. However, wines produced in partial fermentations with *Saccharomyces* spp. have been judged of better quality than wines fermented only with this genus [29]. Furthermore, use of a *Schizosaccharomyces malidevorans* mutant strain produced wines that lacked obvious organoleptic defects [25].

This study examines the potential of the metabolism of *Schizosaccharomyces pombe* 938 applied to white wines with high malic acid levels.

Materials and methods

Microorganisms

The yeast strains used in this study were *Schizo. pombe* 938 from the *Instituto de Fermentaciones Industriales* collection (IFI, CSIC, Madrid, Spain) and *Saccharomyces cerevisiae Cru Blanc* (Maurivin, Melbourne, Australia).

Fermentations

All fermentations were prepared using the must from *Vitis vinifera L.*cv. Airen grapes grown in San Martín de Valdeiglesias (Madrid, Spain). Using a method similar to that described by [32], 3.5 L of unpasteurised must was placed in 4.9-L glass fermentation vessels, leaving enough space for carbon dioxide emission. Sulphur dioxide (60 mg/L) (Panreac, Barcelona, Spain) was added to each, along with 3 g/L malic acid (Panreac, Barcelona, Spain), ensuring at a final concentration of 5 g/L. The sugar content was '226' g/L, relative density 1096 at 15 °C and pH 3.1.

Four assays were performed (all in triplicate): 1) inoculation of the must with solely *Schizo. pombe* 938, 2) inoculation of the must with solely *Sacch. cerevisiae Cru Blanc*, 3) inoculation of the must with *Schizo. pombe* 938 and *Sacch. cerevisiae Cru Blanc* (mixed fermentation) and 4) inoculation of the must with *Schizo. pombe* 938 followed by *Sacch. cerevisiae Cru Blanc* 48 h later. *Schizo. pombe* 938 for these inoculations involved the use of 50 mL of this culture that was grown in sterile must until it reached a population of 10^6 cfu/mL; all 25 g/HL *Saccharomyces cerevisiae Cru Blanc* was added to all fermentations in which this yeast was involved, following the manufacturer's recommendations.

All fermentation processes were carried out at 25 °C. Once fermentation of sugars was complete (deemed to be represented by a remaining glucose + fructose concentration of <2 g/L), the wines were racked and stabilised during 7 days at 4 °C and the final product was bottled. Corked bottles were placed horizontally in a climate chamber TR2V120 (La Sommelière, Saint-Saturnin, France) with conditions of constant temperature and humidity (18 °C and 70 % relative humidity). These conditions were maintained for 1 month until the sensory evaluation took place.

Analytical determination: non-volatile compounds

Glucose + fructose, malic acid, lactic acid, acetic acid, glycerol, citric acid, primary amino nitrogen, urea and

pyruvic acid were all determined using a Y15 Enzymatic Autoanalyzer (Biosystems, Barcelona, Spain). These analyses were performed using the appropriate Biosystems kits (http://www.biosystems.es), except for the pyruvic acid, which was determined using a kit from Megazyme (Bry, Ireland).

pH, dry extract, total acidity, alcohol content and relative density were determined following the methods set out in the Compendium of International Methods of Analysis of Wines and Musts [33].

Analytical determinations: volatile compounds

The changes in the concentration of 18 volatile compounds (acetaldehyde, methanol, *n*-propanol, diacetyl, ethyl acetate, isobutanol, *n*-butanol, 2-butanol, amylic alcohol, isoamylic alcohol, isobutyl acetate, ethyl butyrate, ethyl lactate, *n*-hexanol, isoamyl acetate, 2-phenylethyl alcohol, 2-phenylethyl acetate and 2,3-butanediol), all of which influence wine quality, were monitored by gas chromatography using an Agilent Technologies 6850 gas chromatograph with a flame ionisation detector (Hewlett Packard, Palo Alto, CA, USA). The apparatus was calibrated with a 4-methyl-2-pentanol internal standard. Gas chromatography quality compounds (Fluka, Sigma–Aldrich Corp., Buchs SG, Switzerland) were used to provide standard patterns. Higher alcohols were separated as described in the Official Methods for the Analysis of Musts and Wines [33]. The detection limit was 0.1 mg/l.

Sensorial analysis

The final wines were assessed (blind) by a panel of 10 experienced wine tasters, all members of the staff of the Food Technology Department at the *Universidad Politécnica de Madrid*. Assessment took place in standard sensory analysis chambers with separate booths. Prior to the creation of a consistent terminology by consensus, two visual descriptors, five aromas and four taste attributes were chosen to describe the wines. Formal assessment consisted of two sessions held on different days. The panellists used a 10-cm unstructured scale, from 0 (no defect) to 100 (very strong perceptible defect), to rate the intensity of 12 attributes (Fig. 4).

Statistical analysis

Means and standard deviations were calculated and ANOVA performed using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). Significance was set at $p < 0.05$ for the ANOVA matrix F value. The multiple range test was used to compare means.

Results and discussion

Fermentative kinetics

Figure 1 shows the fermentation kinetics and variations in different variables. Figure 1a shows the change in the relative density during the fermentation period. All fermentation kinetics finished properly, and all ended around day 10. All wines presented a residual sugar concentration of <1.5 g/L (Table 1) and with relative densities around 990 at 20 °C (Fig. 1a). The data show that *Schizo. pombe*, whether alone or combined, were able to consume sugars similar to *Sacch. cerevisiae* alone. *Schizosaccharomyces spp*. has been described as a high fermentative power yeast [5], although in certain assays they showed slower fermentation kinetics [15].

Biological deacidification of wine

Figure 1b, c and d shows the changes of acetic and malic acid contents and pH in the must. Fermentations with *Sacch. cerevisiae Cru Blanc* alone showed a reduction in malic acid of 19.40 %, to reach a final concentration of 4.02 g/L. These data agree with that reported by other authors [5, 15, 34–37] who report that malic acid can be metabolised by species other than *Schizosaccharomyces* although they only reduce its presence by some 25 %. In fermentations involving *Schizo. pombe* 938, nearly all the malic acid was consumed (Fig. 1b). This also agrees with other authors who recorded 75–100 % reductions in malic acid by *Schizosaccharomyces spp*. [17, 21, 25, 29, 38–40]. This reduction largely occurred over the first 4–5 days of fermentation. The final malic acid concentrations recorded were 0.04, 0.04 and 0.06 g/L for only *Schizo. pombe* 938, in the sequential fermentations and mixed fermentation, respectively. There was a malic acid deacidification of around 99 %, with this reduction evident in the pH (Fig. 1c). A twofold decrease in total acidity value in the wines fermented with *Schizo. pombe 938* was observed compared to the samples fermented with *Sacch. cerevisiae Cru Blanc* alone (Table 1). No significant differences were seen in terms of the malic acid concentration at the end of alcoholic fermentation in any of the assays involving *Schizo. pombe* 938, which showed a pH increase of 0.38–0.40 compared to the purely *Sacch. cerevisiae Crue Blanc* fermentations. Therefore, the interest in *Schizosaccharomyces spp*. as biological deacidifiers is proved in this study.

Schizosaccharomyces spp. is usually related to high productions of acetic acid in fermentations at laboratory scale [15]. This is its major drawback at least for the unselected strains commonly used in wine research. However, some authors report satisfactory results for
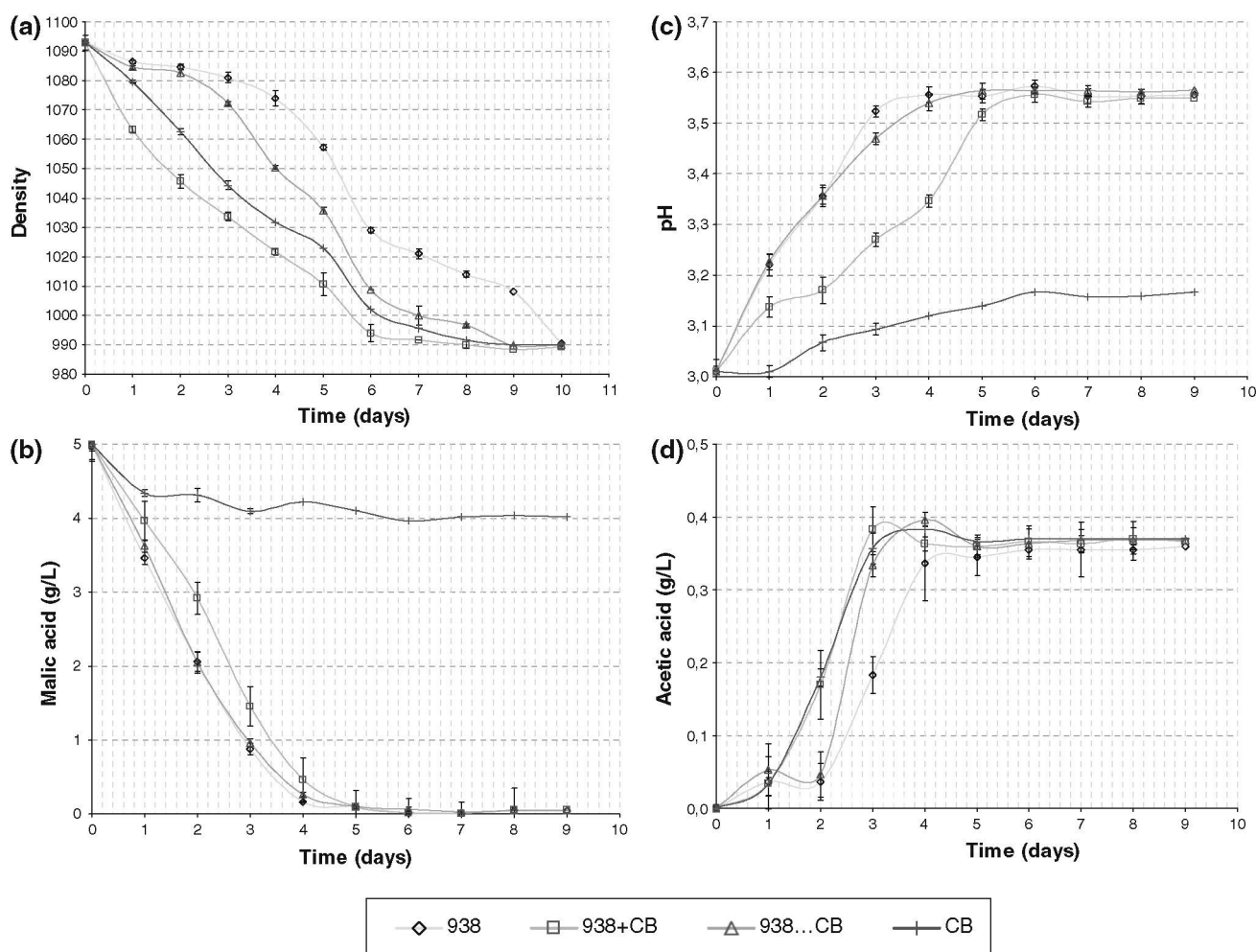
Fig. 1 **a** Change in relative density. **b** Consumption of malic acid. **c** Change in pH. **d** Acetic acid production. Points are mean ± standard deviation. Legend. 938 = fermentation with *Schizo. pombe* 938 alone; 938 + CB = fermentation with *Schizo. pombe* 938 + *Sacch.* *cerevisiae* CB (i.e. mixed fermentation); 938 ... CB = fermentation with *Schizo. pombe* 938 followed by *Sacch. cerevisiae* CB (i.e. sequential fermentation); CB = fermentation with *Sacch. cerevisiae* CB alone

combined fermentations with other yeasts such as *Saccharomyces ssp.*, obtaining wines with no olfactory defects [21, 25]. In this study, the acetic acid concentration stabilised on the third to fourth day of fermentation. The acetic acid content remained at around 0.37 g/L in all *Schizo. pombe* 938 fermentations (Fig. 1d). The selection of *Schizosaccharomyces* strains with low production of acetic acid could bring a new oenological tool for unbalanced musts. This may help remove the spoilage stigma attached to this species.

Fermentation products

Figure 2 shows the pyruvic acid concentration evolution over fermentation. The maximum concentration was reached on the second day in all fermentations, followed by a progressive reduction. The maximum values reached by *Schizo. pombe* 938 alone, in sequential fermentations with

Sacch. cerevisiae Cru Blanc, in mixed fermentations and with *Sacch. cerevisiae* Crue Blanc alone were 0.33, 0.28, 0.16 and 0.11 g/L, respectively. Other studies involving *Schizosaccharomyces* strain fermentations in pasteurised media, values up to 0.39 g/L, were recorded [15]. The pyruvic acid levels in fermentations involving *Schizo. pombe* were always over 0.16 g/L. These values are high compared to others recorded in previous studies for selected *Sacch. cerevisiae* strains and nowadays used in winemaking due to their special ability to produce high levels of pyruvic acid, but in this case only a maximum pyruvic acid value of 0.13 g/L was reached [41]. Non-*Saccharomyces* yeast with high pyruvate production might therefore be of interest in terms of pigment production and stability, especially in red wines [8]. These data could also help to explain their higher glyceropyruvic pathway, as described by other authors during the first stages of the alcoholic fermentation [5].

Assays	Lactic Acid (g/L)	Acetic Acid (g/L)	Malic Acid (g/L)	Gluc + Fruct (g/L)	Citric Acid (g/L)	Glycerol (g/L)	Urea (g/L)	Total Acidity (g/L)	Alcohol (% v/v)	pH
Schizo. pombe 938	0.03 ± 0.02 a	0.35 ± 0.02 a	0.04 ± 0.01 a	0.85 ± 0.37 b	0.39 ± 0.03a	7.12 ± 0.11 b	0.14 ± 0.02a	2.90 ± 0.03 a	12.53 ± 0.02 a	3.56 ± 0.022b
Schizo. pombe 938 + *Sacch. cerevisiae* CB	0.01 ± 0.01 a	0.37 ± 0.03 a	0.06 ± 0.02 a	0.35 ± 0.14 a	0.40 ± 0.02a	6.77 ± 0.28 b	0.36 ± 0.03b	3.06 ± 0.17 a	13.06 ± 0.08 c	3.57 ± 0.04b
Schizo. pombe 938 followed by *Sacch. cerevisiae* CB	0.03 ± 0.02 a	0.36 ± 0.03 a	0.04 ± 0.01 a	1.07 ± 0.10 b	0.39 ± 0.03a	6.98 ± 0.15 b	0.16 ± 0.02a	2.95 ± 0.06 a	12.77 ± 0.03 b	3.55 ± 0.02b
Sacch. cerevisiae CB	0.01 ± 0.01 a	0.37 ± 0.01 a	4.02 ± 0.14 c	0.78 ± 0.29 ab	0.40 ± 0.02a	6.52 ± 0.12 a	1.22 ± 0.09c	6.88 ± 0.07 c	13.18 ± 0.03 c	3.17 ± 0.03a

Results represent mean ± SD for three replicates. Means in the same column with the same letter are not significantly different ($p < 0.05$)
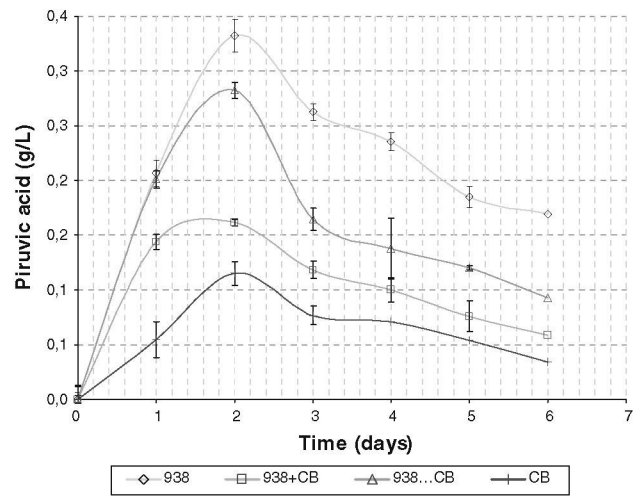

Fig. 2 Pyruvic acid production. Points are mean ± standard deviation. Legend as for Fig. 1
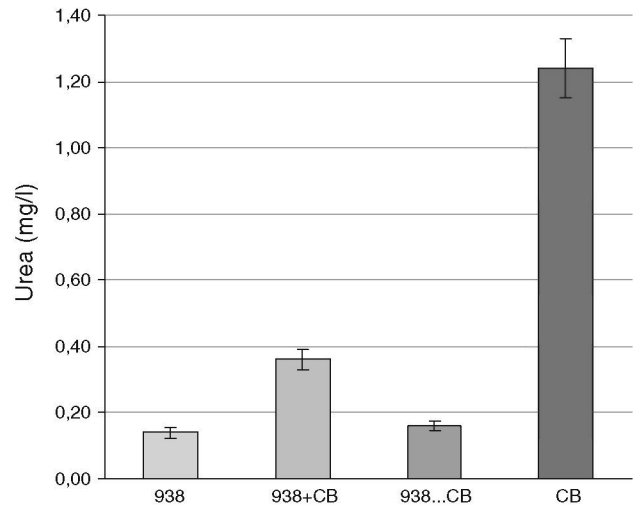

Fig. 3 Urea concentration (mean ± standard deviation) of the final wines. Legend as for Fig. 1

The urea content of the finished wines (Fig. 3) was 0.14 mg/L for that made with only *Schizo. pombe* 938, 0.16 mg/L for that made with the sequential fermentation, 0.36 mg/L for that made with the mixed fermentation and 1.22 mg/L for that made with *S. cerevisiae Cru Blanc* alone. The differences between the fermentations were significant ($p < 0.05$) (Table 1). These differences can be easily explained due to the urease activity of *Schizo. pombe* [14]. A reduction in urea generally leads to lower ethyl carbamate (toxic compound) content because it is its main precursor [42]; the use of *Schizo. pombe* 938 could, therefore, improve wine safety from a toxicological point of view. This strain may also be of interest as it would reduce the possibility of lactic acid bacteria growing by removing malic acid (another of nutrient source), thus reducing the risk of biogenic amine formation [40, 43, 44].

The glycerol content of the wines fermented with *Schizo. pombe* 938 was higher than those recorded in the wine produced with *Saccharomyces cerevisiae Cru Blanc*. These differences were statistically significant ($p < 0.05$) and are in line with other results which show a highly developed glyceropyruvic pathway for *Schizo. pombe*, possibly explaining its greater production of pyruvic acid and glycerine compared to other yeasts [5].

The final alcohol content of the wines made in fermentations involving *Schizo. pombe* 938 was lower than those fermented by *Sacch. cerevisiae Cru Blanc* alone (Table 1). This confirms the findings of other researches who reported that *Schizo. pombe* was a relatively poor producer of alcohol from sugar [45]. The sugar consumption could also be used to produce compounds other than ethanol or to increase the yeast biomass.

Volatile compounds

Table 2 shows production of volatile compounds in the different fermentations. Significant differences were seen in acetaldehyde production. Higher amounts of this compound were recorded in the wines fermented with *Schizo. Pombe* 938, both alone or combined with *Saccharomyces*, than those fermented with *Saccharomyces Cru Blanc* alone. Methanol production (36–40 mg/L) never exceeded the legal limit for white wines [46]. At the end of

fermentation, no significant differences were shown in terms of ethyl acetate concentration. These values were <70 mg/L and can therefore be considered to be acceptable [47]. Higher alcohols (2-methyl-1-butanol and 3-methyl-1-butanol) were produced in moderate quantities in all fermentations, although significantly higher values were reached when *Sacch. cerevisiae Crue Blanc* was involved. The formation of 2-phenylethanol was moderate, although slightly more was obtained in the fermentation which only involved *Sacch. cerevisiae Cru Blanc*, a factor which may have increased its 'floral' aroma. However, there were no significant differences between the mixed inoculations in 2-phenylethanol contents. Significantly more isoamyl acetate was produced in the fermentation involving *Sacch. cerevisiae Cru Blanc* alone. Ethyl lactate production ranged from 6.86 to 17.75 mg/L, recording the highest value when *Schizo. pombe* 938 was used alone.

Sensorial evaluation

Figure 4 shows 'spider web' diagram for the average scores of 12 aromas and taste attributes. Fermentation with *Sacch. cerevisiae Cru Blanc* alone produced the strongest sensations of acidity. This is directly correlated with the final levels of total acidity and malic acid described in Table 1. No differences were found between the tasters regarding oxidation, reduction, acetic acidity and hue.

Table 2 Volatile compounds (mg/L) detected in the different fermentations involving the use of *Schizosaccharomyces pombe* 938 and *Saccharomyces cerevisiae Cru Blanc* (CB) alone or combined (all performed at 25 °C with an initial sugar concentration of 226 g/L and initial malic acid content of 5 g/L)

Compounds	*Schizo. pombe* 938	*Schizo. pombe* 938 + *Sacch. cerevisiae* CB	*Schizo. pombe* 938 followed by *Sacch. cerevisiae* CB	*Sacch. cerevisiae* CB
Acetaldehyde	27.10 ± 2.39 b	23.22 ± 2.18 b	26.21 ± 2.48 b	16.21 ± 1.26 a
Methanol	36.59 ± 1.09 a	44.42 ± 8. 639 a	43.91 ± 7.43 a	40.01 ± 6.80 a
1-Propanol	20.91 ± 0.85 ab	22.35 ± 0.61 b	22.18 ± 1.79 b	19.61 ± 0.95 a
Diacetyl	5.40 ± 1.40 b	2.12 ± 1.34 a	2.56 ± 1.03 a	2.12 ± 0.12 a
Ethyl acetate	29.88 ± 3.35 a	29.25 ± 1.90 a	30.23 ± 3.54 a	27.11 ± 0.95 a
2-Butanol	nd	nd	nd	nd
Isobutanol	21.30 ± 0.47 a	35.93 ± 1.63 a	31.87 ± 2.71 a	38.38 ± 1.65 a
1-Butanol	nd	nd	nd	nd
2-Methyl-1-butanol	70.45 ± 3.75 a	98.77 ± 6.72 c	85.86 ± 5.26 b	109.92 ± 5.24 c
3-Methyl-1-butanol	26.60 ± 4.72 a	37.03 ± 5.78 b	33.96 ± 1.23 ab	35.90 ± 5.07 b
Isobutyl acetate	nd	nd	nd	nd
Ethyl butyrate	nd	nd	nd	nd
Ethyl lactate	17.75 ± 1.98 c	8.83 ± 0.17 ab	12.86 ± 0.48 b	6.86 ± 0.05 a
2-3 Butanediol	853.02 ± 38.80 b	779.36 ± 36.28 b	810.18 ± 29.78 b	517.9 ± 24.04 a
Isoamyl acetate	3.50 ± 0.30 a	3.63 ± 0.39 ab	3.51 ± 0.38 a	4.47 ± 0.76 b
Hexanol	4.12 ± 0.07 a	4.552 ± 0.13 b	4.397 ± 0.10 b	4.480 ± 0.08 b
2-Phenylethanol	30.58 ± 7.92 a	46.71 ± 2.77 bc	42.23 ± 2.26 b	52.54 ± 5.95 c
Phenylethyl acetate	5.25 ± 0.20 a	5.97 ± 0.12 b	5.93 ± 0.12 b	5.99 ± 0.13 b

Results represent the mean ± SD for three replicates. Means in the same row with the same letter are not significantly different ($p < 0.05$)

Fig. 4 Taste and aroma attribute scores for the final wines produced in the different fermentations involving the use of *Schizosaccharomyces pombe* 938 and *Saccharomyces Cerevisiae* Cru Blanc (CB) alone or combined (all performed at 25 °C with an initial sugar concentration of 226 g/L and initial malic acid content of 5 g/L)... Legend. 938 = fermentation with *Schizo. pombe* 938 alone; 938 + CB = fermentation with *Schizo. pombe* 938 + *Sacch. cerevisiae* CB (i.e. mixed fermentation); 938 ... CB = fermentation with *Schizo. pombe* 938 followed by *Sacch. cerevisiae* CB (i.e. sequential fermentation); CB = fermentation with *Sacch. cerevisiae* CB alone
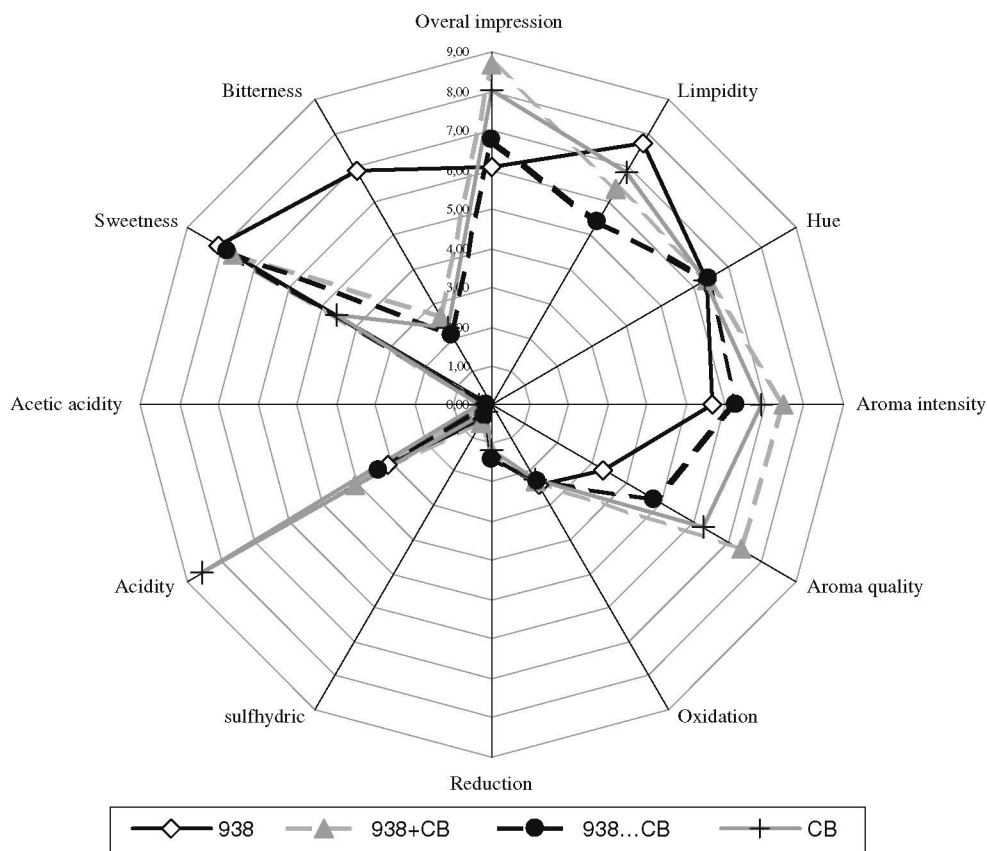
None of the wines that involved fermentation with *Schizo. pombe* 938 had any perceptible organoleptic problems, although a sensation of bitterness was higher in the case of fermentation with *Schizo. pombe* 938 alone. The mixed fermentation received the best scores from all tasters followed by fermentation with *Sacch. cerevisiae Cru Blanc* alone.

Conclusions

This study shows that *Schizo. pombe* 938 meets the basic requirements for a white winemaking yeast that can be applied as a resource to solve technical problems, especially in musts with high malic acid content. The major drawback of this genus, strong acetic acid production, was solved in this study due to its correct management in mixed and sequential fermentation between *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* that minimised the level of volatile acidity. Other strains could be studied to see which might be selected in terms of their ability for malic deacidification, acetic acid production, pyruvic acid production, their volatile compound profile and lower amounts of urea production. *Schizosaccharomyces spp.* appears to be an alternative to be used in combination with *Saccharomyces cerevisiae* in order to produce less standardised wines for the market.

The high pyruvic acid production of yeast strains such us *Schizossaccharomyces pombe* 938 could improve the formation of stable pigments in red wines. Finally, yeast strains with high urease activity can be used as a new tool to assure wine safety due to the breakdown of ethyl carbamate precursors.

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