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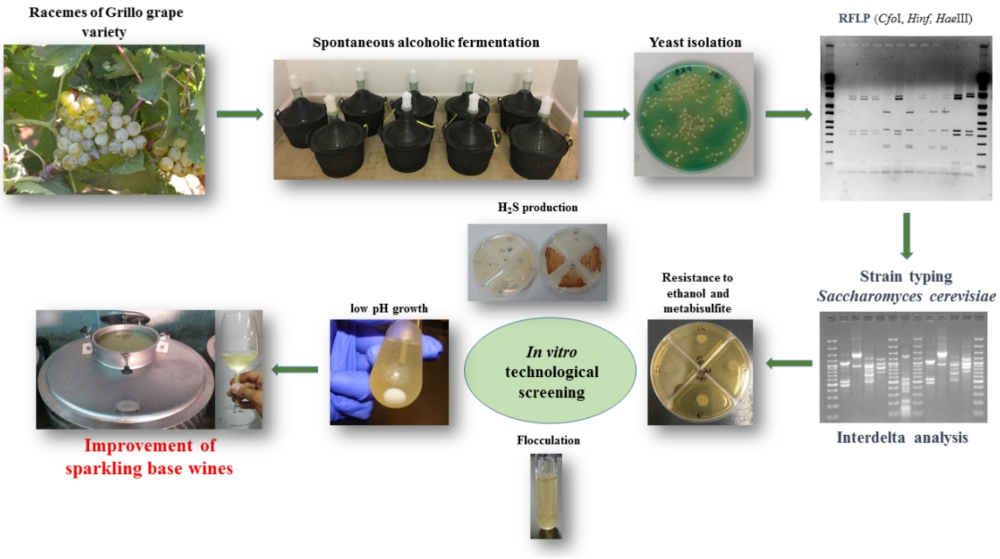

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Diversity of *Saccharomyces cerevisiae* strains associated to racemes of Grillo grape variety

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One-sentence Summary: The selection of *Saccharomyces cerevisiae* strains suitable for fermenting must with extremely low pH (<3) and high acidity values will improve the characteristics of the sparkling base wine.

Keywords: Raceme; *Saccharomyces cerevisiae*; sparkling wine; oenological selection; alcoholic fermentation; yeast starter

ABSTRACT

The most important oenological characteristics of high quality sparkling wines are high content of acidity and low pH. Racemes are late-maturing grapes of Grillo variety characterized by low pH and high content of tartaric and malic acids and, due to their intrinsic characteristics, might represent an interesting technological solution to increase acid quality of base sparkling wine. To this purpose, the use of yeasts able to ferment grape must at very low pH is mandatory for the success of the process. In this work, 261 *Saccharomyces cerevisiae* isolated from spontaneous vinifications of Grillo grape racemes were subject to intraspecific characterization by interdelta analysis which evidenced a total population consisting of 82 strains which were screened for their basis of technological traits including SO_2 and alcohol tolerance, flocculence, growth at low temperatures and qualitative features such as H_2S production. Eleven strains with interesting technological performance in vitro were inoculated into musts obtained from racemes of Grillo grape variety and microfermentation were monitored. For the first time an ecological investigation of yeast associated to raceme grapes has been carried out and provided an innovative strategy to improve the acidity of a Sicilian sparkling base wine from Grillo grape variety.

INTRODUCTION

Sicily (Southern Italy) represents the fourth Italian area for wine production (ISMEA 2019). The Sicilian wines are significantly different in relation to the production area, soil and climatic conditions, processing techniques and grape variety characteristics. Currently, in Sicily 31 wine enjoy recognition of quality such as protected denomination of origin (PDO) and protected geographic indication (PGI) (ISMEA 2017). Due to the orographic characteristics, Sicily can be considered a miniature continent, with an incredible diversity of areas intended for wine production. The qualitative aspects of wine are influenced by the composition of the must and by the microbial community carrying out the alcoholic fermentation. The final quality of a wine is undoubtedly determined through its aroma (Rapp and Mandery 1986; Selli et al. 2006; Styger *et al.* 2011) characterized by a complexity of volatile organic compounds (VOCs) (Selli *et al*. 2004; Sánchez-Palomo *et al.* 2005; Muñoz-González *et al.* 2011). The presence and diversity of VOCs depends on grape variety and secondary products synthesized during alcoholic fermentation (Lund and Bohlmann 2006; Mauriello *et al.* 2009; Katarína *et al.* 2014). During alcoholic fermentation yeasts convert sugars into ethanol and synthesize higher alcohols, esters and fatty acids having a significant impact on the sensory aspect of the resulting wine (Lambrechts and Pretorius 2000; Procopio *et al.* 2011). Biosynthetic capacity of yeasts is species and strain-dependent (Zamora 2009). The use of a few commercial *Saccharomyces cerevisiae* starter strains has led to a flattening of the aromatic complexity of wines. To overcome this drawback, several wineries selected indigenous yeasts and used them as autochthonous starter strains (Van der Westhuizen 2000; Vaudano *et al.* 2019).

In the last years, high attention was paid in Sicily to the production of sparkling wines. The varieties mainly used to this purpose are Catarratto and Grillo, two indigenous grape varieties cultivated for decades in the island (Francesca *et al*. 2011). However, the musts to be used for the production of sparkling wines have to be characterized by high values of total acidity, malic acid and pH, extremely important factors for the production of quality and longevity sparkling wines (Garofalo *et* *al.* 2016; Di Gianvito et al. 2018). The dry-Mediterranean climate of Sicily does not allow the production of musts with optimal acidity parameters for the production of sparkling base wines. The problem is circumvented through the early harvest of the grapes or by collecting the grapes in high mountain production areas which delays the ripening process of the grapes. Catarratto and Grillo variety have the property of producing naturally racemes. The grape racemes are the bunch that are formed by the lateral shoots of the vine deriving from the ready buds (Pastena 1990). Some varieties like Grillo produce a high number of racemes (Pastena 1990). The racemes are not generally harvested, but due to their high acidity, they could be employed for the production of base wines to be destined for sparkling wine production.

In the present research, we report the first microbiological characterization of raceme grapes focused on the isolation, characterization and selection of *S. cerevisiae* strains. Specifically, the following objectives were pursued: (i), to isolate yeasts from raceme grape of Grillo cultivar; (ii), to characterize genetically and technologically the strains of *S. cerevisiae*; (iii), to monitor physical-chemical parameters during the microvinification of pasteurized raceme grape must.

MATERIALS AND METHODS

Sample collection

Six vineyards of the “Grillo” variety were sampled for grapes and berries within different wine production areas of Sicily (Italy) during the harvesting of two consecutive vintages (2018 and 2019). All vineyards were at least 20 km far from the closest winery. The sampling was carried out according to the methodology reported by Settanni *et al.* (2012). Grape samples (G) were placed into sterile plastic bags containing an isotonic peptone solution (10 g/L Bacto Soytone, 2 ml/l Tween 80) and were incubated at 30 °C for 3 h to collect the microorganisms hosted on peel surface (Renouf *et al.* 2005).

Berries were crushed by stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 5 min at the highest speed to obtain must that was transferred into sterile flasks (5 l-volume) and maintained at 10 and 15°C until total sugar consumption. The samples collected for analysis were: grape must just pressed (M1), must at 1/5 (M2), 3/5 (M3) and 5/5 (M4) of sugar consumption.

Microbiological analysis

Cell suspensions of grape and must samples were serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy). Decimal dilutions were spread plated (0.1 ml) onto Wallerstein laboratory (WL) nutrient agar (Oxoid, Milano, Italia), incubated at 28 °C for 48-72 h, for total yeasts (TY) and onto modified ethanol sulfite agar (MESA), prepared as reported by Francesca et al. (2010), incubated at 28 °C for 72 h, to detect presumptive *Saccharomyces* spp. (PS). Both media were supplemented with chloramphenicol (0.5 g/l) and byphenil (1 g/l) to inhibit the growth of bacteria and moulds, respectively. Analyses were carried out in duplicate.

Yeasts were isolated from both growth media used for counts. At least three colonies per morphology were collected from the differential medium WL, while 10 colonies were randomly picked up from MESA. All isolates were purified to homogeneity after several sub-culturing steps onto WL and at least two isolates (from each sample) sharing the same morphology were subjected to the genetic characterization.

Yeast identification

The DNA extraction was performed using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

In order to perform a first differentiation of yeasts, all selected isolates were analysed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1 and ITS4 (Esteve-Zarzoso *et al.* 1999) by means of T1 Thermocycler (Biometra, Göttingen, Germany) and subsequently the amplicons were digested by three endonucleases [*Cfo*I, *Hae*III and

*Hinf*I (MBI Fermentas, St. Leon-Rot, Germany)] at 37 °C for 8 h. The bands of ITS amplicons and restriction fragments were visualized on agarose gel in two steps: first on agarose gel (1.5% w/v) and subsequently in 3% agarose gel in 1 × TBE (89 mmol/l Tris-borate, 2 mmol/l EDTA pH 8) buffer (Gaglio *et al.* 2017). The gels were stained, visualized and acquired as described by Francesca et al. (2015). Standard DNA ladders were such as those reported by Gaglio *et al.* (2017). All isolates, belonging to group presumptively identified as *S. cerevisiae* were further processed by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. D1/D2 region was amplified with primers NL1 and NL4 (Kurtzman and Robnett, 1998). PCR products were visualised as above. DNA sequencing reactions were performed at AGRIVET (University of Palermo, Italy). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

Strain typing of *Saccharomyces cerevisiae* isolates

The intraspecific characterization of the isolates belonging to *S. cerevisiae* species was carried out by interdelta analysis with primers delta12 and delta 21 (Legras and Karst 2003; Sannino *et al.* 2013; Moschetti *et al.* 2016). The PCR products were analyzed on agarose gel 2.0% (w/v) in 1 × TBE buffer and visualized as above reported.

Technological screening of *Saccharomyces cerevisiae* strains

In vitro tests

All strains belonging to the species *S. cerevisiae* were evaluated for their potential in winemaking. The ability to produce H_2S was tested using a qualitative method performed on Bismuth Sulfite Glucose Glycerin Yeast extract (BiGGY) agar (Oxoid) (Jiranek et *al.*1995). H_2S was estimated by colony blackening after 3 days of incubation at 28 °C. A five-level scale was used for colour evaluation: 0 = white, 1 = beige, 2 = light brown, 3 = brown, 4 = dark brown, 5 = black. The resistance to various levels of ethanol (from 12 to 16 % v/v) and potassium metabisulphite (KMBS) (from 150 to 250 mg/l) were determined onto MESA. *S. cerevisiae* GR1 (Francesca *et al.* 2010) and NF213, belonging to the culture collection of Department of Agricultural, Food and Forestry Sciences (University of Palermo, Italy), producing low amount of H_2S and resistant to high levels of KMBS and ethanol were used as control strains. The growth at low temperatures and low pH was determined in Yeast Extract Peptone Dextrose (YPD) broth at 10 and 15 °C and pH 2.5, 2.8 and 3.0 for five days. Growth patterns were examined through visual inspection of samples through a light microscope (Carl Zeiss Ltd) (Pretorius 2000; Di Maio *et al.* 2012).

The growth pattern was examined also by flocculation measurement as reported by Bidard et *al.* (1995) and Tofalo *et al.* (2016). All analysis were carried out in triplicate.

Microfermentation tests

The strains showing the best technological performances (low production of H_2S, resistance to ethanol, KMBS, ability to grow at low pH and low temperatures) were evaluated for their ability to ferment a grape must from racemes. Broth cultures in the stationary phase were washed twice in Ringer's solution and inoculated at a final concentration of about 106 CFU/ml in 1 L of pasteurized Grillo grape must of racemes added with KMBS (100 mg/L).

Grape raceme must showed the following chemical composition: pH 2.9; total reducing sugars 149.27 g/l; total acidity 18.44 g/l (tartaric acid); malic acid 12.17 g/l; total SO_2 90 mg/l; free SO_2 31 mg/l, and yeast assimilable nitrogen (YAN) 177.23 mg/l.

Microfermentations were carried out at 10 and 15 °C. In order to allow CO_2 removal, the flasks were plugged with a Müller valve containing sulphuric acid (Ciani and Rosini 1987) and the weight loss was monitored until a daily decrease below 0.01 g (end of fermentation process). According to Ciani and Maccarelli (1998), the fermentation power (FP) was evaluated as the ethanol amount (% v/v) produced at the end of the process, the fermentation rate (FR) was calculated as CO_2 daily produced and the fermentation purity (FPu) was calculated as acetic acid (g/L) per ethanol (% v/v) produced at the end of micro-fermentation. At the end of fermentation, the wines were analysed for residual sugar, acetic acid and glycerol content following the standard methods of the Organization of Vine and Wine.

Statistical analysis

The results of yeast population concentrations and chemical-physical parameters of micro-fermentations were processed statistically by analysis of variance (ANOVA) and homogeneous groups were identified by Tukey's test. Principal component analysis (PCA) were performed by XLSTAT Version 2014.5.03 (Addinsoft, USA). A symmetric biplot of PCA was constructed based on chemical-physical parameters monitored during microfermentation.

RESULTS

Microbiological analysis of spontaneous fermentations of grape must racemes

The viable counts of total yeasts (TY) and presumptive *Saccharomyces* (PS) populations analysed from grape racemes showed average values in the range 4.93 - 5.04 Log CFU/g and 2.20 - 2.26 Log CFU/g, respectively (Tab. 1).

Yeast populations were also monitored at the different steps during sugar consumption. TY load of M1 samples were higher than that detected on the corresponding grapes. During fermentation, both TY and PS counts increased significantly until concentration of about 7.36 (G2) and 5.42 (G3) Log CFU/ml at 5/5 of sugar consumption.

In general, the effect of vineyard, year and sample type showed significant effect ($P<0.05$) on count data registered for TY populations, whereas the concentration levels of PS were affected by degree of consumption of sugars at M3 and M4.

Isolation, identification and typing of *S. cerevisiae* strains

A total of 1174 colonies from WL and 487 from MESA were isolated, purified to homogeneity and separated on the basis of colony morphology on WL. At least three cultures from each sample were selected for a total of 402 isolates (278 from WL and 124 from MESA) which were subjected to molecular identification. After analysis of 5.8S-ITS amplicons, all isolates were clustered into main groups: the isolates associated to cluster I (n. 261), showing size of 5.8S-ITS region comprised between 800 and 900 bp, were presumptively identified as *Saccharomyces* spp. (White *et al.* 1990); all isolates grouped into cluster II (n. 141) with size of ITS amplicons different from range 800-900 bp were considered non-*Saccharomyces* yeasts. All isolates of group I were further investigated by restriction analysis of 5.8S-ITS region and they were directly identified by comparison of restriction bands with those available in literature (Esteve-Zarzoso *et al.* 1999; Cordero *et al.* 2011; Muccilli *et al.* 2011). The profiles obtained by digestion of amplification products with C*fo*I resulted in two restriction bands (380 and 340 pb). Four bands (320, 225, 180 and 125 bp) were obtained with *Hae*III, while three restriction bands of 370, 355 and 110 bp were obtained when amplicons were digested with *Hinf*I endonuclease. All isolates belonging to the species *S. cerevisiae* were genetically typed. The interdelta analysis was able to separate the isolates into 82 groups (Fig.1) as indicated by Guarcello et al. (2019) the 3% of isolates was is enough to determine species by sequencing of D1/D2 domain of the 26S rRNA gene that successful confirmed the species identification (Accession numbers: MT093501-03).

Technological screening of *S. cerevisiae* strains

The 82 *S. cerevisiae* strains were screened for their oenological traits (Tab. 2). Eighty-one strains were characterized by a low production of H_2S on Biggy agar plates (white - brown colony); 71 strains showed growth in presence of high concentrations (12-14-16 % v/v) of ethanol. Only 14 strains were also able to grow in presence of high concentrations of KMBS (150-250 mg/L) and ethanol (16% v/v). The growth at two different temperatures (10 ° and 15 ° C) and at a pH of 2.5 was positive for all 82 strains, whereas barely 13 yeasts showed flocculent growth.

From the previous technological tests, 11 strains were selected and used as starters to ferment grape must at 10 and 15 °C in presence of 100 mg/L of KMBS. The results of the fermentation kinetics are reported in Table 3. The results of the different parameters monitored varied with the starter strains. The FP values of the microfermentations conducted at 10°C (8.50-9.73) were lower than those registered at 15 °C (12.80-13.63). The highest values were displayed by the strain MSE4 at 10 °C (9.73), and the strains MSE92 and MSE106 at 15 °C (13.63, 13.56, respectively).

The highest values of FR (T= 10°C) were observed for the strains MSE105 and MSE106, both at 3 (0.33-0.34) and 12 d (11.04-11.90) of fermentation. In addition, the strain MSE106 at 15°C and 3 d of alcoholic fermentation showed the highest FR value (7.01). The strains MSE35 and MSE92 showed the highest FR values at 15°C and after 12 d of alcoholic fermentation with values of 21.15 and 20.88, respectively.

The volatile acidity (VA) at the end of alcoholic fermentation for both temperatures showed values in the range 0.17-0.67 g/l acetic acid. The lowest values of VA were observed in wines obtained with the strains MSE105 (0.17 g/l at 10°C and 0.29 g/l at 15°C) and MSE106 (0.18 g/l at 10°C).

Not statistically significant values for $P<0.05$ were observed for Fpu and pH values.

The lowest residual sugar values were registered for the must inoculated with the strain MSE92 at both fermentation temperatures (10°C-15°C). The values of glycerol were between 5.18 and 7.57 g/l. The highest glycerol values were observed for the strains MSE4 (5.40-7.67 g/l) and MSE30 (5.39-7.64 g/l) at both temperatures.

The results of PCA are reported in Fig. 2. The biplot graph explained 64.45% of the total variability for microfermentations conducted at 10°C (Fig. 2A). In detail, the strains MSE6, MSE13, MSE40, MSE 105 and MSE106 were correlated with FR (3 and 12 d). The strains MSE30, MSE35, MSE41 and MSE92 were associated with GL, while, the strain MSE 4 was found to be related to FP, FPu and VA. On the other hand, the strain MSE27 was found to be closely related to RS. For the microfermentations conducted at 15°C, biplot graph explained 68.27% of the total variability (Fig. 2B). The strains MSE4 and MSE92 were statistically correlated with GL, FP, FR (3 d) and pH, while the strains MSE30, MSE35 and MSE41 were associated to Fpu, VA and FR (12 d). On the third quadrant of biplot, the strains MSE6, MSE13 and MSE40 resulted related to RS.

DISCUSSION

The present study dealt with the isolation of *S. cerevisiae* from racemes of Grillo variety grapes, quite widespread in the viticulture areas of western Sicily (Trapani, Italy). The must obtained from the racemes has physicochemical characteristics (pH, organic acids) that make it suitable for the production of base sparkling wines. In Sicily, due to climatic conditions, the wines obtained from grapes show low values of acidity, sapidity and minerality, important parameters for obtaining a good sparkling wine base. To overcome this problem, the early harvesting of grapes (pre-harvest) is practiced. But very often, the acidic profile and the sugar content do not allow suitable base wines to be used for sparkling wine making. For this reason, racemes might represent a valuable source to obtain wines with acidity values 4-5 times higher than those obtained with the early harvesting of the grapes (Margalyt 2012). Thus, these wines could be used as "blending wines" to improve the acidic and aromatic profile of the sparkling wine bases. However, raceme must represents a hostile environment for a regular alcoholic fermentation process, mainly due to pH values < 3, total acidity values of 18-20 g/l and high content in malic acid and tartaric acid. The search for starter strains suitable for fermenting racemic musts with extreme physicochemical characteristics is necessary to apply this strategy.

To our knowledge, this is the first study showing the molecular, physiological and technological characterization of indigenous *S. cerevisiae* strains isolated from Grillo grape racemes. Both in the racemes, before pressing, and in the spontaneously fermented must *S. cerevisiae* constituted about 50-70% of the total blastomycetic populations. Consequently, it seems that the incidence of isolates of *S. cerevisiae* is equal to that of bunches of grapes originating from primary shoots (Cappello *et al.* 2004). Molecular analysis allowed the differentiation of *S. cereviasiae* isolates into 82 polymorphic groups showing the high variability of *S. cerevisiae* in relation to pedo-climatic conditions, agronomic practices, harvest period and viticulture areas. These results are in line with the results reported by Schuller and Casal (2007), who analyzed the populations of *S. cerevisiae* in vineyards in the same region for three consecutive years and showed a high variability Other authors claim that the diversity of *S. cerevisiae* isolates is related to the isolation location of the vineyard (González et *al.* 2020). However, isolation of *S. cerevisiae* strains in our study occurred at different levels of sugar consumption during alcoholic fermentation. In fact, from *in vitro* technological screening, several strains were excluded for the microfermentation tests because they were poorly resistant to high concentrations of ethanol and metabisulphite. The isolates of *S. cerevisiae* represented a genetically heterogeneous group of yeasts naturally adapted to live in hostile environments. The best strains are endowed with physiological characteristics that allowed their tolerance to high concentrations of ethanol and in presence of SO_2. They also showed a medium-low production of H_2S and a moderate biosynthesis of ethanol. During alcoholic fermentation, volatile acidity values were low. These technological traits could be a starting point for further studies aimed to valorize *S. cerevisiae* biodiversity. Moreover, their ability to ferment raceme musts represents an important value for the development of Sicilian sparkling wines overcoming the issues related to the low acidity.

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292 *Conflict of interest.* None declared.

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FIGURE LEGENDS

Figure 1. Dendrogram of *Saccharomyces cerevisiae* strains obtained by Interdelta analysis.

Figure 2. Biplot graph showing the distribution of different yeast strains in relation to the physico-chemical parameters detected during microfermentations at 10°C **(A)** and 15°C **(B).** Abbreviations: FR (3 d), fermentation rate at 3 days; FR (12 d), fermentation rate at 12 days; FP, fermentation purity; FPu, fermentation purity; GL, glycerol content; VA, volatile acidity; RS, residual sugar.

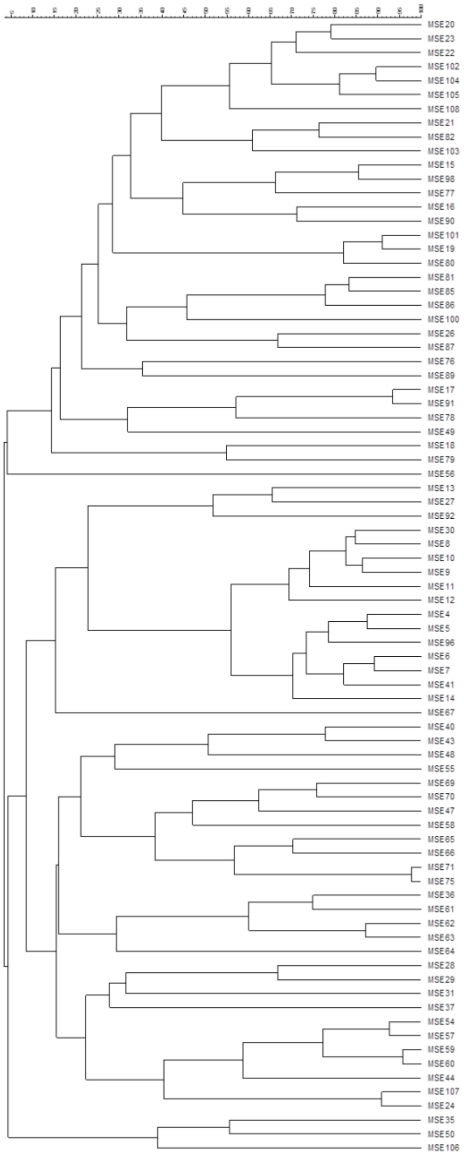

Figure 1. Dendrogram of Saccharomyces cerevisiae strains obtained by Interdelta analysis.

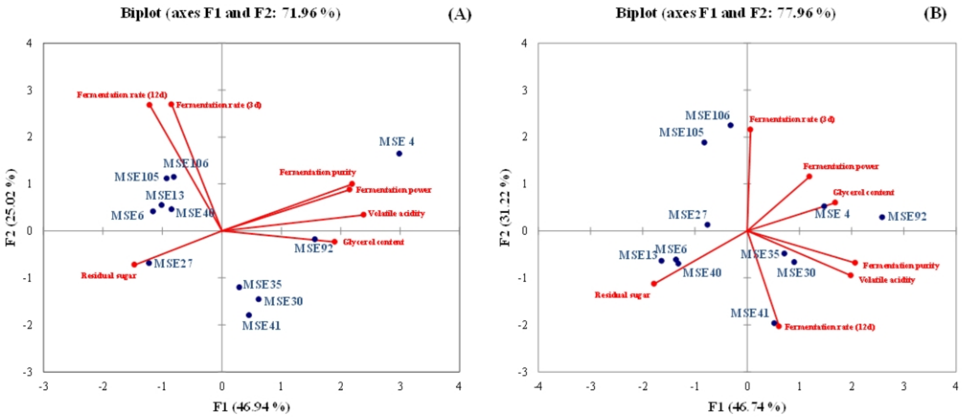

Figure 2. Biplot graph showing the distribution of different yeast strains in relation to the physico-chemical parameters detected during microfermentations at 10°C (A) and 15°C (B). Abbreviations: FR (3 d), fermentation rate at 3 days; FR (12 d), fermentation rate at 12 days; FP, fermentation purity; FPu, fermentation purity; GL, glycerol content; VA, volatile acidity; RS, residual sugar.

Table 1. Microbial concentration of yeast populations isolated from grape racemes and must in spontaneous fermentation at different levels of sugar consumption.

Samples	**Microbial loads**						**Statistical significance**
	G1	**G2**	**G3**	**G4**	**G5**	**G6**	
Total yeasts							
Winemaking process							
Grape berries	4.93±0.05^{ab}	4.89±0.05^{b}	4.96±0.05^{ab}	4.95±0.06^{ab}	5.01±0.05^{ab}	5.04±0.05^{a}	*
M1	5.53±0.23^{a}	5.05±0.22^{a}	5.03±0.23^{a}	5.16±0.19^{a}	5.38±0.20^{a}	5.05±0.22^{a}	N.S.
M2	6.04±0.31^{a}	5.69±0.30^{ab}	5.76±0.27^{ab}	5.81±0.28^{ab}	5.21±0.33^{b}	5.91±0.30^{ab}	*
M3	6.38±0.29^{a}	5.83±0.26^{a}	6.03±0.29^{a}	6.11±0.26^{a}	5.75±0.31^{a}	6.46±0.27^{a}	N.S.
M4	6.81±0.32^{a}	7.36±0.42^{a}	7.08±0.35^{a}	6.63±0.33^{a}	7.30±0.37^{a}	6.51±0.39^{a}	N.S.
Presumptive *Saccharomyces* spp.							
Winemaking process							
Grape berries	2.25±0.04^{a}	2.22±0.02^{a}	2.23±0.05^{a}	2.20±0.02^{a}	2.26±0.04a	2.23±0.07^{a}	N.S.
M1	3.56±0.21^{a}	3.56±0.26^{a}	3.49±0.24^{a}	3.72±0.24^{a}	3.97±0.18^{a}	4.03±0.19^{a}	N.S.
M2	4.07±0.34^{a}	4.11±0.31^{a}	4.47±0.31^{a}	4.29±0.32^{a}	4.18±0.34^{a}	4.37±0.31^{a}	N.S.
M3	4.13±0.21^{b}	4.50±0.22^{ab}	4.79±0.23^{a}	4.64±0.21^{ab}	4.58±0.17^{ab}	4.49±0.27^{ab}	*
M4	4.71±0.25^{b}	4.46±0.24^{b}	5.42±0.24^{a}	4.91±0.25^{ab}	4.89±0.23^{ab}	4.69±0.18^{b}	*

Results indicate average values ± standard deviation of three plate counts. Log CFU/g for grape berries and log CFU/ml for must.
Abbreviations: M1, grape must just pressed; M2, must at 1/5 of sugar consumption; M3, must a 3/5 of sugar consumption; M4, must a 5/5 of sugar consumption
P value: *, $P < 0.05$; N.S., not significant.
Data within a line followed by the same letter are not significantly different according to Tukey's test.

Table 2. Technological screening of *Saccharomyces cerevisiae* strains.

Strain code	H_2S[a]	Ethanol[b]	KMBS[c]	10 °C[d]	15 °C[e]	Growth Pattern[f]	Strain code	H_2S[a]	Ethanol[b]	KMBS[c]	10 °C[d]	15 °C[e]	Growth Pattern[f]
MSE 4	3	3	2	+	+	S	MSE 56	1	3	1	+	+	S
MSE 5	3	3	1	+	+	F	MSE 57	1	3	0	+	+	S
MSE 6	3	3	1	+	+	S	MSE 58	1	2	0	+	+	S
MSE 7	3	3	1	+	+	S	MSE 59	3	3	0	+	+	S
MSE 8	3	3	2	+	+	S	MSE 60	1	0	0	+	+	S
MSE 9	3	3	2	+	+	S	MSE 61	3	3	1	+	+	S
MSE 10	3	3	2	+	+	S	MSE 62	1	3	1	+	+	S
MSE 11	3	3	2	+	+	F	MSE 63	2	3	1	+	+	S
MSE 12	3	3	2	+	+	S	MSE 64	3	3	1	+	+	F
MSE 13	3	3	2	+	+	S	MSE 65	2	0	0	+	+	S
MSE 14	2	3	0	+	+	S	MSE 66	3	3	1	+	+	S
MSE 15	3	3	1	+	+	S	MSE 67	3	3	1	+	+	S
MSE 16	3	3	0	+	+	S	MSE 69	3	3	1	+	+	S
MSE 17	3	3	0	+	+	S	MSE 70	3	3	1	+	+	S
MSE 18	3	1	0	+	+	S	MSE 71	0	3	0	+	+	S
MSE 19	3	3	1	+	+	S	MSE 75	0	3	0	+	+	S
MSE 20	1	3	0	+	+	S	MSE 76	3	3	0	+	+	S
MSE 21	3	3	1	+	+	S	MSE 77	3	3	0	+	+	S
MSE 22	3	3	0	+	+	S	MSE 78	3	1	0	+	+	S
MSE 23	3	3	2	+	+	S	MSE 79	3	1	1	+	+	F
MSE 24	3	3	0	+	+	S	MSE 80	4	3	0	+	+	S
MSE 26	3	3	2	+	+	S	MSE 81	3	1	0	+	+	S
MSE 27	1	3	1	+	+	F	MSE 82	3	0	0	+	+	S
MSE 28	2	3	1	+	+	S	MSE 85	2	1	0	+	+	S
MSE 29	3	3	1	+	+	F	MSE 86	3	3	1	+	+	S
MSE 30	3	3	2	+	+	S	MSE 87	3	3	1	+	+	S
MSE 31	3	3	1	+	+	F	MSE 89	3	3	1	+	+	S
MSE 35	3	3	1	+	+	F	MSE 90	3	3	0	+	+	S
MSE 36	3	3	0	+	+	F	MSE 91	2	3	0	+	+	S
MSE 37	3	3	1	+	+	S	MSE 92	2	3	2	+	+	S
MSE 40	1	3	1	+	+	S	MSE 96	2	3	0	+	+	F
MSE 41	3	3	2	+	+	F	MSE 98	2	3	1	+	+	S
MSE 43	3	3	1	+	+	S	MSE 100	3	3	2	+	+	S
MSE 44	3	3	1	+	+	S	MSE 101	1	3	1	+	+	S
MSE 47	3	2	0	+	+	S	MSE 102	0	3	0	+	+	S
MSE 48	3	3	1	+	+	S	MSE 103	0	3	1	+	+	S
MSE 49	0	3	0	+	+	S	MSE 104	1	3	1	+	+	S
MSE 50	0	3	0	+	+	S	MSE 105	1	3	2	+	+	S
MSE 53	0	0	0	+	+	S	MSE 106	3	3	1	+	+	F
MSE 54	0	3	1	+	+	S	MSE 107	3	3	1	+	+	F
MSE 55	1	3	0	+	+	S	MSE 108	3	3	1	+	+	S

[a] color of colony on Biggy agar plates: 0 = white; 1 = beige; 2 = light brown; 3 = brown; 4 = dark brown; 5 = black.

[b] 0, 0%(v/v); 1, 12% (v/v); 2, 14% (v/v); 3, 16% (v/v) of ethanol contained in MESA plates at which strains showed growth.

[c] 0, no growth in the plates; 1, 15 g/hl of MBSK + 16% ethanol; 2, 25 g/hl of MBSK + 16% (v/v) of ethanol contained into MESA plates at which strains showed growth.

[d] +, growth; , not growth at 10 °C in YPD broth.

[e] +, growth; , not growth at 15 °C in YPD broth.

[f] S, suspended growth; F, flocculant growth in YPD broth.

Table 3. Kinetics of alcoholic micro-fermentations of preselected *Saccharomyces cerevisiae* strains.

Strain code	pH[a]		Fermentation power[b]		Fermentation rate[c1]		Fermentation rate[c2]		Volatile acidity[d]		Fermentation purity[e]		Residual sugar[f]		Glycerol content[g]	
	10 °C	15°C	10 °C	15 °C	10 °C	15 °C	10 °C	15 °C	10 °C	15 °C	10 °C	15 °C	10 °C	15 °C	10 °C	15 °C
MSE 4	2.94±0.01^{a}	2.93±0.02^{a}	9.73±0.18^{a}	13.45±0.16^{ab}	0.25±0.16^{ab}	6.17±0.13^{c}	9.82±0.10^{d}	20.02±0.09^{c}	0.65±0.15^{a}	0.53±0.12^{ab}	0.06±0.01^{a}	0.04±0.02^{a}	68.22±1.47^{e}	3.46±0.10^{e}	5.40±0.12^{a}	7.67±0.15^{a}
MSE6	2.84±0.02^{a}	2.85±0.01^{a}	8.48±0.11^{d}	13.10±0.160^{ab}	0.27±0.10^{ab}	5.09±0.10^{c}	10.40±0.10^{c}	20.39±0.06^{b}	0.27±0.12^{ab}	0.33±0.10^{ab}	0.03±0.01^{ab}	0.02±0.01^{a}	88.38±1.13^{bc}	5.45±0.06^{a}	5.18±0.06^{ab}	6.66±0.12^{c}
MSE13	2.97±0.03^{a}	2.97±0.03^{a}	8.52±0.15^{d}	13.12±0.09^{bc}	0.28±0.13^{ab}	5.13±0.07^{de}	10.64±0.12^{c}	20.55±0.05^{b}	0.28±0.16^{ab}	0.36±0.12^{ab}	0.03±0.01^{ab}	0.02±0.01^{a}	87.26±1.31^{bc}	5.29±0.01^{a}	5.21±0.08^{ab}	6.78±0.14^{c}
MSE27	2.90±0.01^{a}	2.90±0.01^{a}	8.75±0.16^{d}	12.80±0.19^{c}	0.01±0.00^{b}	6.28±0.18^{c}	9.99±0.14^{d}	19.47±0.12^{d}	0.25±0.14^{ab}	0.43±0.13^{ab}	0.02±0.01^{b}	0.02±0.01^{a}	84.98±1.39^{c}	4.31±0.02^{c}	5.09±0.09^{b}	7.22±0.16^{b}
MSE30	2.90±0.02^{a}	2.89±0.01^{a}	8.95±0.18^{bcd}	13.10±0.13^{bc}	0.02±0.01^{b}	5.27±0.12^{d}	6.90±0.15^{g}	20.30±0.14^{b}	0.34±0.14^{ab}	0.58±0.14^{ab}	0.03±0.01^{ab}	0.04±0.02^{a}	91.14±1.57^{ab}	4.62±0.09^{b}	5.39±0.11^{a}	7.64±0.14^{a}
MSE35	2.93±0.01^{a}	2.92±0.02^{a}	8.92±0.17^{bcd}	13.05±0.13^{bc}	0.01±0.00^{b}	4.95±0.06^{e}	7.43±0.16^{f}	21.15±0.10^{a}	0.29±0.14^{ab}	0.50±0.14^{ab}	0.02±0.01^{b}	0.03±0.01^{a}	65.30±1.38^{e}	3.31±0.03^{e}	5.30±0.11^{ab}	7.52±0.13^{ab}
MSE40	2.95±0.03^{a}	2.93±0.02^{a}	8.50±0.05^{d}	13.08±0.07^{bc}	0.28±0.08^{ab}	5.09±0.08^{c}	10.41±0.10^{c}	20.54±0.04^{b}	0.29±0.10^{ab}	0.38±0.16^{ab}	0.03±0.01^{ab}	0.02±0.01^{a}	86.98±1.36^{bc}	5.31±0.08^{a}	5.25±0.07^{ab}	6.81±0.11^{c}
MSE41	2.92±0.01^{a}	2.90±0.00^{a}	8.83±0.17^{cd}	12.92±0.14^{c}	0.03±0.01^{b}	2.25±0.21^{f}	5.72±0.13^{h}	20.42±0.07^{b}	0.34±0.14^{ab}	0.53±0.14^{ab}	0.03±0.01^{ab}	0.04±0.01^{a}	91.53±1.47^{a}	4.64±0.13^{b}	5.34±0.10^{ab}	7.57±0.15^{ab}
MSE92	2.92±0.03^{a}	2.91±0.02^{a}	9.32±0.16^{ab}	13.63±0.13^{a}	0.01±0.00^{b}	6.31±0.06^{c}	8.25±0.13^{e}	20.88±0.09^{a}	0.39±0.17^{ab}	0.67±0.14^{a}	0.04±0.02^{ab}	0.05±0.02^{a}	51.49±1.48^{f}	2.61±0.02^{f}	5.27±0.09^{ab}	7.47±0.14^{ab}
MSE105	2.91±0.02^{a}	2.90±0.02^{a}	8.89±0.18^{bcd}	13.00±0.14^{c}	0.33±0.15^{a}	6.66±0.08^{b}	11.90±0.15^{a}	16.26±0.08^{f}	0.17±0.12^{b}	0.29±0.11^{b}	0.02±0.01^{b}	0.02±0.01^{a}	76.61±1.35^{d}	3.88±0.17^{d}	5.32±0.10^{ab}	7.54±0.13^{ab}
MSE106	2.92 ±0.01^{a}	2.91±0.01^{a}	9.27±0.17^{abc}	13.56±0.16^{a}	0.34±0.17^{a}	7.01±0.02^{a}	11.04±0.16^{a}	17.01±0.11^{e}	0.18±0.16^{b}	0.32±0.13^{ab}	0.02±0.01^{b}	0.02±0.01^{a}	76.32±1.40^{d}	3.87±0.14^{d}	5.22±0.11^{ab}	7.40±0.15^{ab}
Statistical analysis	N.S.	N.S.	***	**	**	***	***	**	*	*	*	N.S.	***	***	*	**

[a] pH values determined at the end of alcoholic fermentation.
[b] Ethanol (% v/v) produced at day 12 of microfermentation.
[c1] CO$_2$ produced at day 3 of fermentation.
[c2] CO$_2$ produced at day 12 of fermentation.
[d] Acetic acid (g/l) produced at day 12 of microfermentation.
[e] Volatile acidity formed in relationship to ethanol produced at day 12 of microfermentation.
[f] Reducing sugars (g/l) at day 12 of microfermentation.
[g] Glycerol (g/l) produced at day 12 of microfermentation
Results indicate mean values ± SD of two measurements. P value: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; N.S., not significant.
Data within a line followed by the same letter are not significantly different according to Tukey's test.