J. Gen. Appl. Microbiol., **48**, 261–267 (2002)

Full Paper

Isolation and clonal pre-selection of enological *Saccharomyces*

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(Received February 25, 2002; Accepted September 20, 2002)

The aim of the present study was to perform a fast pre-selection from a great number of wine yeasts using a simple phenotypic-based methodology that allows many different strains to be simultaneously tested. A total of 150 elliptic yeasts, isolated from must and wine from black grapes of a distinctive Italian variety, were studied. Yeasts were identified to genus level by assessing their ability to ferment glucose and their production of spores on acetate agar. The *Saccharomyces* strains were seeded on BiGGY agar to determine their H_2S production, on calcium carbonate agar to test their acetic acid production, and on grape-skin agar and on grape-seed agar to assess their interaction with phenolic compounds. The *Saccharomyces* strains were also examined for fermentative vigor after 2 d or 7 d both with and without the addition of 100 mg L^{-1} of SO_2 in must at 20° brix and pH 3.20. At the end of fermentation, the wines produced by the 18 best yeasts were analyzed and the strains were studied for additional biochemical and technological characteristics. The resistance of the strains to simultaneous acid-stress and osmotic-stress was studied carrying out in duplicate winemaking tests in must at 30° brix and pH 2.60. A remarkable heterogeneity among the 150 autochthonous yeasts studied was demonstrated. The phenotypical biodiversity is particularly interesting for several technological characteristics useful in winemaking, such as fermentation vigor, acetic acid production and malic acid content of the wines. The vast majority of the elliptic wine yeasts isolated did not show suitable characteristics, so only 18 strains, 12% of the total, remained for the final tests. Many of the strains that had passed the preliminary screenings revealed some defects when they were studied for fermentation performance, both in standard winemaking and under stressors. Two strains exhibited particularly interesting performances: one strain for winemaking of normal musts and the other for winemaking of musts from dried grapes or under stressful conditions.

Key Words——phenotypical method; *Saccharomyces*; stress resistance; wine yeast pre-selection

Introduction

The selection of wine yeasts for enological use was traditionally carried out on the basis of their technological and quality-linked phenotypical characteristics (Giudici and Zambonelli, 1992). For this purpose different methodologies were designed. Recently a two-step procedure was proposed: a pre-selection based on resistance to SO_2, killer activity, growth at high temperature and low foam production, followed by a selection based on volatile acidity, ethanol production, and residual sugars (Regodon et al., 1997). Another methodology based on phenotypical characteristics has four consecutive stages: (1) fermenting capacity of the strains under secondary fermentation conditions; (2) formation of volatile acidity, resistance to SO_2, for-

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mation of H_2S, flocculation capacity and adherence to glass; (3) autolytic capacity of the yeast; (4) foaming properties of the autolysates obtained (Martinez-Rodriguez et al., 2001). Alternatively, using specifically designed genetic selection procedures, strains showing potentially interesting phenotypes were isolated from the natural populations (Nadal et al., 1996, 1999).

The aim of the present study was to quickly perform a pre-selection from a great number of wine yeasts using a simple phenotypic-based methodology that allows many different strains to be simultaneously tested.

Materials and Methods

Yeast isolation. Ten samples of red wines from black grapes of the Calabrian distinctive variety Gaglioppo were collected from ten wineries; all these wines were obtained by spontaneous fermentation. In addition, two batches of freshly extracted must from the same grape variety were collected from two wineries. Each must was fractioned in three lots of 1 L, which were allowed to ferment in thermostat at 4, 25, and 40°C. The 10 wines and the six lots of grape musts (at the start, middle, and end of fermentation) were plated on Sabouraud agar (Biokar Diagnostics, Beauvais, France) and the plates were incubated at 25°C for up to 48–72 h both aerobically and anaerobically, using gas-pack catalysts (Oxoid, Ltd., Hampshire, UK). Representative colonies were observed by phase contrast microscopy Standard 20 (Zeiss, Oberkochen, Germany), and five elliptic yeasts per plate were chosen.

Yeast identification. Yeast isolates were identified to genus level by phenotypical characteristics (Barnett et al., 1990), in order to exclude all the non-*Saccharomyces* yeasts and reduce the number of strains to be checked. The first characteristic examined was the ability to ferment glucose, which was assessed by looking for the formation of gas (CO_2) collected in Durham tubes. Yeast extract medium was used (0.5% w/v of dry yeast extract) with 2% of glucose. Each test tube was inoculated with a fresh yeast suspension and incubated at 25°C for 7 d. Following this, the glucose-fermenting strains were studied in Petri plates for production of spores of the genus *Saccharomyces* on acetate agar at 25°C for 10 d (Fowell, 1952).

Yeast screening. The yeasts were studied in Petri plates for the following biochemical characteristics: H_2S production on BiGGY agar at 25°C for 48 h (Nickerson, 1953), acetic acid production on calcium carbonate agar at 25°C for 7 d (Belarbi and Lemaresquier, 1994), interaction with phenolic compounds on grape-skin agar (triturated grape-skin 200 g L^{-1}, peptone from casein 7.5 g L^{-1}, yeast extract 4.5 g L^{-1}, agar 15 g L^{-1}) and on grape-seed agar (triturated grape-seed 100 g L^{-1}, peptone from casein 7.5 g L^{-1}, yeast extract 4.5 g L^{-1}, agar 15 g L^{-1}) at 25°C for 7 d (Caridi, A. and Cufari, A., unpublished results). The screening media were seeded with a small quantity of yeast biomass, 15–18 strains per plate and, after incubation, the biomass color (white, pale hazel, hazel, dark hazel, black) on BiGGY agar, the presence of haloes on calcium carbonate agar, and the biomass color (white, pale grey, pale hazel, dark hazel) on grape-skin agar and grape-seed agar were observed. The darkness of the color on BiGGY agar is in direct proportion to H_2S production. A halo on calcium carbonate agar indicates strains producing high quantities of acetic acid. On grape-skin agar and grape-seed agar, a white/pale grey biomass is explained as zero or low absorption of phenolic compounds; pale to dark hazel biomass is explained as medium or high absorption of phenolic compounds. The yeasts were also examined for their fermentative vigor after 2 d and 7 d in must from Calabrian black grapes, both with and without the addition of SO_2. The sugar content of the grape must was 20° brix and the pH was 3.20. The must was distributed in flasks in quantities of 100 ml, 10 ml of liquid paraffin was added to avoid the surface coming into contact with oxygen, and the must was then pasteurized at 100°C for 20 min. After pasteurization, one half of the flasks had 100 mg L^{-1} of SO_2 as potassium metabisulphite added. Flasks were inoculated in duplicate with 5 ml of 48 h precultures and incubated at 25°C. After 2 d and 7 d the weight loss caused by CO_2 production was determined; the fermentative vigor was expressed as g of CO_2 100 ml^{-1} of must. Yeast screening was performed to exclude the strains producing high quantities of acetic acid, duplicate copies of those strains with identical activity on phenolic compounds, those with fermentation vigor after 2 d ≤ 4 in must without SO_2 and ≤ 2 in must with the addition of SO_2, and those with fermentation vigor after 7 d ≤ 8 in must both with and without the addition of SO_2.

Yeast selection. The grape musts inoculated to determine the fermentation vigor of the screened strains were analyzed at the end of fermentation, using stan-

dard (Ough and Amerine, 1988) or enzymatic (Boehringer kits) methods for: ethanol, titratable acidity, acetic acid, unitary acetic acid production, glycerol, unitary glycerol production, malic acid, and total SO_2. Data were subjected to basic mathematical and statistical analysis. The screened strains were studied for additional biochemical characteristics. Glucose, galactose, saccharose, maltose, raffinose, melibiose, starch, and D-mannitol fermentation at 25°C, and glucose fermentation at 37°C were studied, by looking for the formation of gas (CO_2) in Durham tubes; for this purpose, yeast extract medium (2% w/v of yeast extract) containing 2% of each compound (or 4% for raffinose) was inoculated with each strain and incubated for 7–21 days. The resistance of the strains to simultaneous acid-stress and osmotic-stress was studied, carrying out in duplicate winemaking tests using grape must adjusted to 30° brix by adding glucose and corrected to pH 2.60 by adding diluted sulphuric acid. At the end of fermentation, wines were analyzed for ethanol content and, only when this value was of at least 9% v/v both with and without the addition of SO_2, wines were analyzed for the other parameters.

Results and Discussion

Yeast identification and screening

A total of 150 elliptic yeasts were isolated from both must and wine. Only 86 isolates were able to ferment glucose with production of gas. Among the 86 glucose-fermenting yeasts, 72% produced spores of the genus *Saccharomyces* on acetate agar, 20% did not produce spores, and 8% produced spores of non-*Saccharomyces* genera (*Dekkera*, *Torulaspora*, and *Zygosaccharomyces*). On the basis of these tests, only one *Saccharomyces* strain with identical characteristics and isolated from the same sample was maintained, the other strains being excluded: thus the number of isolates was reduced to 46.

The 46 isolates of *Saccharomyces* were studied for their behavior on the screening media. Two percent of the strains examined were white on BiGGY agar, 41% were pale hazel, 37% were hazel, 15% were dark hazel, and 5% were black. Only 9% of the strains examined produced a halo on calcium carbonate agar, showing their unsuitability for winemaking. On grape-skin agar 40% of the strains produced a white biomass, 28% pale grey, 26% pale hazel, and only 4% dark hazel; the last category consisting of strains able to subtract the phenolic compounds from grape skin by adsorption on their cell walls. On grape-seed agar 66% of the strains produced a white biomass, 17% pale grey, 15% pale hazel and only 2% dark hazel. This behavior indicated that it is very difficult to find strains of *Saccharomyces* able to subtract phenolic compounds from grape seed by adsorption on their cellular walls. The 46 strains of *Saccharomyces* were grouped in frequency classes on the basis of the fermentation vigor after 2 d and 7 d in grape must at 20° brix with or without SO_2 (Fig. 1). When SO_2 was not added, after 2 d only 19% of the strains showed a fermentative vigor ≤ 2. However, 72% of the strains were included in the third and in the fourth frequency classes, with fermentation vigor >4. In the presence of SO_2, after 2 d 33% of the strains showed a fermentative vigor ≤ 2, while 56% of the strains were included in the third and in the fourth frequency classes, with fermentation vigor >4. After 7 d, in must without the addition of SO_2, 74% of the strains and, in the presence of SO_2, 72% of the strains had fermentation vigor >8 and were included in the third and in the fourth frequency classes.

Yeast selection

Following the results of the screening tests, the number of strains was reduced to 18. These *Saccharomyces* were put into 7 groups (Table 1) according to their fermentation patterns; 10 of them were included in the same group. One strain was unable to ferment the glucose at 37°C, two strains did not ferment the galactose, four the saccharose, two the maltose, five the raffinose, and none the melibiose, the starch or the D-mannitol. The physicochemical parameters of the wines obtained using the 18 screened *Saccharomyces* in winemaking of a batch of must at 20° brix are reported in Table 2. Ethanol, glycerol, unitary glycerol production, and malic acid content did not depend on the presence or absence of SO_2 in must. However, titratable acidity, acetic acid, and unitary acetic acid production were significantly higher ($p<0.01$) in the wines produced from must without SO_2 compared to the wines produced from must with the addition of 100 mg L^{-1} of SO_2. Under simultaneous acid-stress and osmotic-stress, only 8 of the 18 *Saccharomyces* screened were able to produce at least 9% v/v of ethanol both with and without the addition of 100 mg L^{-1} of SO_2. The physicochemical parameters of the wines obtained using these eight screened *Sac-*

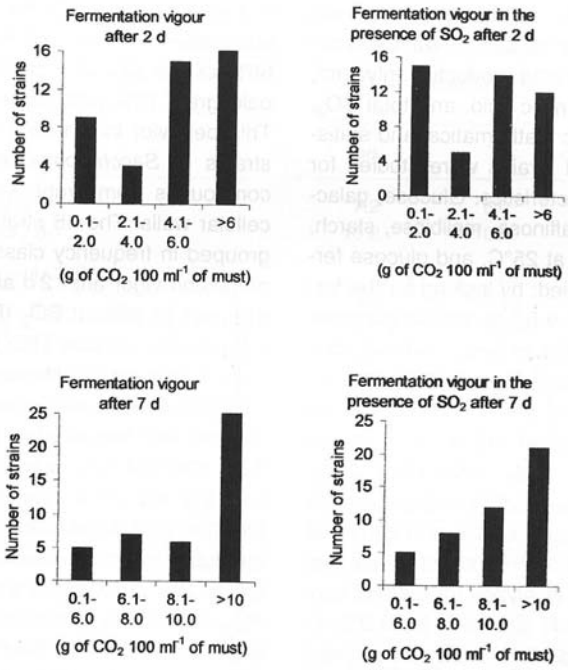

Fig. 1. Distribution of the 46 *Saccharomyces* in frequency classes based on their fermentation vigor after 2 d and 7 d, both with and without the addition of 100 mg L^{-1} of SO_2 in grape must at 20° brix.

Table 1. Fermentation patterns of the 18 selected strains of *Saccharomyces*.

Fermentation patterns	I	II	III	IV	V	VI	VII
Glucose	+	+	+	+	+	+	+
Glucose at 37°C	+	+	+	+	+	−	+
Galactose	+	+	+	+	−	−	+
Saccharose	+	−	+	+	+	+	−
Maltose	+	+	+	−	+	+	−
Raffinose	+	−	−	+	+	+	−
Melibiose	−	−	−	−	−	−	−
Starch	−	−	−	−	−	−	−
D-Mannitol	−	−	−	−	−	−	−
Number of strains	10	3	1	1	1	1	1

charomyces in winemaking of an acidified (pH 2.60) must at 30° brix are reported in Table 3. The malic acid content was significantly higher ($p<0.01$) in the wines produced from must without SO_2 compared to the wines produced from must with the addition of 100 mg L^{-1} of SO_2. The other parameters examined did not show significant differences.

Excluding the strains that produced wines with low ethanol content, high acetic acid production, and high unitary acetic acid production, among the *Saccharomyces* screened, the best strains obtained with the present research were:

Strain 58—Selected for winemaking of normal musts, pale hazel on BiGGY agar, no halo producing on calcium carbonate agar, white on grape-skin agar, pale grey on grape-seed agar, included in fermentation pattern I.

Strain 75—Selected for winemaking of musts from dried grapes or under stressful conditions, pale hazel on BiGGY agar, no halo producing on calcium carbonate agar, white on grape-skin agar and on grape-seed agar, included in fermentation pattern III.

The technological characteristics of these two strains are reported in Table 4. Fermentation vigor after 2 d of strain 75 is partially influenced by SO_2; however, after 7 d this is no longer evident. In grape must at 20° brix (pH 3.20) strain 58 exhibits the best performance, both with and without the addition of SO_2, producing wines with higher values of ethanol, glycerol, unitary glycerol production, and malic acid content and lower values of titratable acidity, acetic acid, and unitary acetic acid production. Similarly, in grape must at 30° brix (pH 2.60) without the addition of

Table 2. Minimum, mean, and maximum of the physicochemical parameters of the wines produced using the 18 selected *Saccharomyces* in must at 20° brix.

Parameter	Must without SO_2			Must with addition of 100 mg L^{-1} of SO_2		
	Minimum	Mean	Maximum	Minimum	Mean	Maximum
Ethanol (% v/v)	10.55	11.27	11.60	11.00	11.36	11.55
Titratable acidity (g L^{-1})	6.660	7.317 A	8.303	6.563	6.910 B	7.463
Acetic acid (g L^{-1})	0.134	0.282 A	0.511	0.049	0.181 B	0.386
Unitary acetic acid production (g 100 ml^{-1} of ethanol)	0.120	0.250 A	0.460	0.040	0.160 B	0.340
Glycerol (g L^{-1})	4.773	6.682	7.739	3.918	6.710	9.187
Unitary glycerol production (g 100 ml^{-1} of ethanol)	4.340	5.930	6.730	3.470	5.910	8.130
Malic acid (g L^{-1})	1.122	1.470	1.748	1.051	1.404	1.689
Total SO_2 (mg L^{-1})	<5	<5	<5	5	26	66

A, B: $p<0.01$ indicate the significance of the comparison.

Table 3. Minimum, mean, and maximum of the physicochemical parameters of the wines produced using 8 selected *Saccharomyces* in acidified (pH 2.60) must at 30° brix.

Parameter	Must without SO_2			Must with addition of 100 mg L^{-1} of SO_2		
	Minimum	Mean	Maximum	Minimum	Mean	Maximum
Ethanol (% v/v)	9.10	9.66	10.30	9.50	9.93	10.60
Titratable acidity (g L^{-1})	8.610	9.006	9.488	8.955	9.046	9.248
Acetic acid (g L^{-1})	0.613	0.831	1.065	0.684	0.856	1.069
Unitary acetic acid production (g 100 ml^{-1} of ethanol)	0.670	0.860	1.150	0.700	0.860	1.100
Glycerol (g L^{-1})	5.959	7.668	9.367	7.587	8.911	11.132
Unitary glycerol production (g 100 ml^{-1} of ethanol)	6.020	7.940	9.190	7.780	8.980	11.360
Malic acid (g L^{-1})	1.748	1.939 A	2.233	1.595	1.689 B	1.784
Total SO_2 (mg L^{-1})	91	121	149	69	113	136

A, B: $p<0.01$ indicate the significance of the comparison.

SO_2, strain 75 exhibits the best performance, producing a wine with higher values of ethanol, glycerol, unitary glycerol production, and malic acid content and lower values of titratable acidity, acetic acid, and unitary acetic acid production.

Perspectives

The use of selected yeasts for winemaking gives clear advantages over the traditional spontaneous fermentation. In these days, many commercial wine yeasts have special purpose, as for good extraction of color, for making new types of wine, or for expression of a variety character. It was recently reconfirmed that autochthonous selected strains are important contributors to wine fermentation (Esteve-Zarzoso et al., 2000). The present study shows a remarkable heterogeneity among the autochthonous yeasts studied. This phenotypical biodiversity is particularly interesting for those technological characteristics which are useful in winemaking, such as fermentation vigor, acetic acid production, and malic acid content of the wines. In particular, a wide variability of the glycerol content, as well as unitary glycerol production, was observed among the strains. This agrees with the results of a recent study, which indicate that achieving high glycerol content in wine requires the selection or improvement of yeast strains rather than the control of growth and cultivation conditions (Remize et al., 2000). The vast ma-

Table 4. Technological characteristics of the two selected *Saccharomyces*, 58 and 75.

Strain	Must without SO_2		Must with addition of 100 mg L^{-1} of SO_2	
	58	75	58	75
Fermentation vigor after 2 d	7.2	6.0	7.1	2.7
Fermentation vigor after 7 d	10.2	10.4	10.4	9.3
Grape must at 20° brix—pH 3.20				
Ethanol (% v/v)	11.60	11.00	11.40	11.30
Titratable acidity (g L^{-1})	6.660	7.545	6.638	7.253
Acetic acid (g L^{-1})	0.192	0.262	0.109	0.325
Unitary acetic acid production (g 100 ml^{-1} of ethanol)	0.170	0.240	0.100	0.290
Glycerol (g L^{-1})	7.159	4.773	8.001	6.787
Unitary glycerol production (g 100 ml^{-1} of ethanol)	6.170	4.340	7.020	6.010
Malic acid (g L^{-1})	1.299	1.122	1.170	1.051
Total SO_2 (mg L^{-1})	<5	<5	39	9
Grape must at 30° brix—pH 2.60				
Ethanol (% v/v)	9.30	10.00	10.30	10.20
Titratable acidity (g L^{-1})	9.488	8.918	8.955	9.150
Acetic acid (g L^{-1})	0.905	0.770	0.816	0.887
Unitary acetic acid production (g 100 ml^{-1} of ethanol)	0.970	0.770	0.790	0.870
Glycerol (g L^{-1})	6.180	7.146	8.663	8.056
Unitary glycerol production (g 100 ml^{-1} of ethanol)	6.650	7.150	8.410	7.900
Malic acid (g L^{-1})	1.796	2.056	1.784	1.784
Total SO_2 (mg L^{-1})	143	149	112	125

jority of the 150 elliptic wine yeasts isolated did not show the required characteristics; so only 18 strains, or 12%, remained for the final tests. This is normal when clonal selection is carried out (Ezeronye and Okerentugba, 2001). Many of the strains that had passed the preliminary screening revealed defects when they were studied for fermentation performance, both in standard winemaking and under stressors. Two strains were particularly interesting: one strain for winemaking of normal musts and the other for winemaking of musts from dried grapes or under stressful conditions. In our opinion, the method reported in this manuscript must be regarded as a pre-selection system, useful to reduce considerably the number of strains, excluding those that reveal defects before the final selection.

Acknowledgments

This work was supported by a grant from the European Community (E.E.C.)—European Agricultural Guidance and Guarantee Fund (F.E.O.G.A.)—and from the Italian Government—Ministero per le Politiche Agricole—for the Research Project POM B35: "Miglioramento e valorizzazione dei vini ottenuti da uve autoctone dell'Italia meridionale attraverso lo studio ed il controllo delle variabili critiche che ne determinano la tipicità sensoriale."

References

Barnett, J. A., Payne, R. W., and Yarrow, D. (1990) Yeasts: Characteristics and Identification, 2nd ed., Cambridge University Press, Cambridge.

Belarbi, M. A. and Lemaresquier, M. H. (1994) La caratterizzazione dei lieviti. *Vignevini*, **21**, 57–59.

Esteve-Zarzoso, B., Gostincar, A., Bobet, R., Uruburu, F., and Querol, A. (2000) Selection and molecular characterization of wine yeasts isolated from the 'El Penedes' area (Spain). *Food Microbiol.*, **17**, 553–562.

Ezeronye, O. U. and Okerentugba, P. O. (2001) Genetic and physiological variants of yeast selected from palm wine. *Mycopathologia*, **152**, 85–89.

Fowell, R. R. (1952) Sodium acetate agar as a sporulation medium for yeasts. *Nature*, **170**, 578.

Giudici, P. and Zambonelli, C. (1992) Criteri di selezione dei lieviti per enologia. *Vignevini*, **19**, 29.

Martinez-Rodriguez, A., Carrascosa, A. V., Barcenilla, J. M., Pozo-Bayon, M. A., and Polo, M. C. (2001) Autolytic capacity and foam analysis as additional criteria for the selection

of yeast strains for sparkling wine production. *Food Microbiol.*, **18**, 183–191.

Nadal, D., Carro, D., Fernandez-Larrea, J., and Pina, B. (1999) Analysis and dynamics of the chromosomal complements of wild sparkling-wine yeast strains. *Appl. Environ. Microbiol.*, **65**, 1688–1695.

Nadal, D., Colomer, B., and Pina, B. (1996) Molecular polymorphism distribution in phenotypically distinct populations of wine yeast strains. *Appl. Environ. Microbiol.*, **62**, 1944–1950.

Nickerson, W. J. (1953) Reduction of inorganic substances by yeast. I. Extracellular reduction of sulphide by species of *Candida*. *J. Infect. Dis.*, **93**, 43–48.

Ough, C. S. and Amerine, M. A. (1988) Methods for Analysis of Musts and Wine, John Wiley and Sons, New York.

Regodon, J. A., Perez, F., Valdes, M. E., Miguel, C. D., and Ramirez, M. (1997) A simple and effective procedure for selection of wine yeast strains. *Food Microbiol.*, **14**, 247–254.

Remize, F., Sablayrolles, J. M., and Dequin, S. (2000) Re-assessment of the influence of yeast strain and environmental factors on glycerol production in wine. *J. Appl. Microbiol.*, **88**, 371–378.