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$_2$S 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

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**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-
mation of H₂S, flocculation capacity and adherence to
glass; (3) autolytic capacity of the yeast; (4) foaming
properties of the autolysates obtained (Martinez-Ro-
driguez et al., 2001). Alternatively, using specifically
designed genetic selection procedures, strains show-
ing 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 al-
lows 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 winer-
ies. 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 anaerobi-
cally, using gas-pack catalysts (Oxoid, Ltd., Hamp-
shire, 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-Saccha-
romyces 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 (CO₂) 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 pro-
duction of spores of the genus Saccharomyces on ac-
etate agar at 25°C for 10 d (Fowell, 1952).

Yeast screening. The yeasts were studied in Petri
plates for the following biochemical characteristics:
H₂S production on BiGGY agar at 25°C for 48 h (Nick-
erson, 1953), acetic acid production on calcium car-
bonate 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 bio-
mass 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 H₂S pro-
duction. 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 ex-
plained as medium or high absorption of phenolic com-
pounds. The yeasts were also examined for their fer-
mentative vigor after 2 d and 7 d in must from Cal-
abrian black grapes, both with and without the addition
of SO₂. 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 SO₂ as potassium metabisul-
phite 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 CO₂ production
was determined; the fermentative vigor was expressed
as g of CO₂ 100 ml⁻¹ of must. Yeast screening was
performed to exclude the strains producing high quan-
tities 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
SO₂ and ≤2 in must with the addition of SO₂, and
those with fermentation vigor after 7 d ≥8 in must both
with and without the addition of SO₂.

Yeast selection. The grape musts inoculated to de-
termine the fermentation vigor of the screened strains
were analyzed at the end of fermentation, using stan-
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\textsubscript{2} (Fig. 1). When SO\textsubscript{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\textsubscript{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\textsubscript{2}, 74% of the strains and, in the presence of SO\textsubscript{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\textsubscript{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\textsubscript{2} compared to the wines produced from must with the addition of 100 mg L\textsuperscript{-1} of SO\textsubscript{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\textsuperscript{-1} of SO\textsubscript{2}. The physicochemical parameters of the wines obtained using these eight screened Sac-
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

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

<table>
<thead>
<tr>
<th>Fermentation patterns</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose at 37°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saccharose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of strains</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
SO₂, 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 2. Minimum, mean, and maximum of the physicochemical parameters of the wines produced using the 18 selected *Saccharomyces* in must at 20° brix.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Must without SO₂</th>
<th>Must with addition of 100 mg L⁻¹ of SO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (% v/v)</td>
<td>Minimum Mean Maximum</td>
<td>Minimum Mean Maximum</td>
</tr>
<tr>
<td></td>
<td>10.55 11.27 11.60</td>
<td>11.00 11.36 11.55</td>
</tr>
<tr>
<td>Titratable acidity (g L⁻¹)</td>
<td>6.660 7.317 A 8.303</td>
<td>6.563 6.910 B 7.463</td>
</tr>
<tr>
<td>Acetic acid (g L⁻¹)</td>
<td>0.134 0.282 A 0.511</td>
<td>0.049 0.181 B 0.386</td>
</tr>
<tr>
<td>Unitary acetic acid production (g 100 ml⁻¹ of ethanol)</td>
<td>0.120 0.250 A 0.460</td>
<td>0.040 0.160 B 0.340</td>
</tr>
<tr>
<td>Glycerol (g L⁻¹)</td>
<td>4.773 6.682 7.739</td>
<td>3.918 6.710 9.187</td>
</tr>
<tr>
<td>Unitary glycerol production (g 100 ml⁻¹ of ethanol)</td>
<td>4.340 5.930 6.730</td>
<td>3.470 5.910 8.130</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>1.122 1.470 1.748</td>
<td>1.051 1.404 1.689</td>
</tr>
<tr>
<td>Total SO₂ (mg L⁻¹)</td>
<td>&lt;5 &lt;5 &lt;5</td>
<td>5 26 66</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Must without SO₂</th>
<th>Must with addition of 100 mg L⁻¹ of SO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (% v/v)</td>
<td>Minimum Mean Maximum</td>
<td>Minimum Mean Maximum</td>
</tr>
<tr>
<td></td>
<td>9.10 9.66 10.30</td>
<td>9.50 9.93 10.60</td>
</tr>
<tr>
<td>Acetic acid (g L⁻¹)</td>
<td>0.613 0.831 1.065</td>
<td>0.684 0.856 1.069</td>
</tr>
<tr>
<td>Unitary acetic acid production (g 100 ml⁻¹ of ethanol)</td>
<td>0.670 0.860 1.150</td>
<td>0.700 0.860 1.100</td>
</tr>
<tr>
<td>Glycerol (g L⁻¹)</td>
<td>5.959 7.668 9.367</td>
<td>7.587 8.911 11.132</td>
</tr>
<tr>
<td>Unitary glycerol production (g 100 ml⁻¹ of ethanol)</td>
<td>6.020 7.940 9.190</td>
<td>7.780 8.980 11.360</td>
</tr>
<tr>
<td>Malic acid (g L⁻¹)</td>
<td>1.748 1.939 A 2.233</td>
<td>1.595 1.689 B 1.784</td>
</tr>
<tr>
<td>Total SO₂ (mg L⁻¹)</td>
<td>91 121 149</td>
<td>69 113 136</td>
</tr>
</tbody>
</table>

A, B: *p*<0.01 indicate the significance of the comparison.
The majority 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


