

Exploitation of technological variability among wild non-*Saccharomyces* yeasts to select mixed starters for the production of low alcohol wines

A. Grazia, A. Pietrafesa, A. Capece, R. Pietrafesa, G. Siesto, and P. Romano

Università degli Studi della Basilicata, Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Viale dell'Ateneo Lucano 10, 85100 Potenza (PZ)

Abstract. Increase of the sugar content in grape must, and consequently, alcohol levels in wine are some of the principal problems affecting the winemaking industry. High alcohol content can compromise wine quality, creating sensory imbalances, as well as decreasing the perception of some flavors. The technological approaches proposed at this aim, although allowing achievement of the purpose, can determine negative influence on quality of wine. A promising strategy is based on the use of specific microorganisms, such as selected yeast strains, mainly non-*Saccharomyces*, able to convert grape must sugars towards secondary metabolites rather than ethanol. This study aims at screening of wild non-*Saccharomyces* strains in order to identify those suitable for the use in mixed starter for the production of wine with reduced alcohol content and, at the same time, with improved aromatic characteristics.

1. Introduction

In recent decades there has been an increase in the alcohol content of wine mainly linked to climate changes, which have led to the production of grapes with high sugar content and consequently the production of wines with high level of ethanol.

High alcohol content not only compromises wine quality (increasing hotness and bitterness perception) [1], but also represents an economic and social problem. This is related both to taxes imposed in some countries for the higher alcohol drinks and to demands of modern consumers, preferring wines with a low percentage of ethanol and fruity favour. To meet consumer expectations, winemaking industry is focused on the production of wine with a moderate ethanol level and a peculiar organoleptic profile [2].

The technological approaches, proposed at this aim, include the use of strategies in vineyard (optimization of the harvest date to obtain a reduction of sugar in grape), application of pre-fermentation or winemaking practices (removal of sugar from grape must) and post-fermentation practices (distillation, nanofiltration, reverse osmosis). Although those techniques allow achieving this purpose, they can have a negative influence on wine's quality of wine [3].

To avoid this inconvenience, research aims to apply biotechnological approaches, principally based on the selection of new starter cultures possessing specific technological properties, such as lower sugar-alcohol conversion and high enzymatic activity to improve organoleptic quality.

In this context, non-*Saccharomyces* yeasts deserve special attention. Yeasts "non-*Saccharomyces*" include different genera and species present in the early stages

of spontaneous fermentation. Generally, they are not very tolerant to high ethanol level and unable to complete the fermentation process. They were considered in the past as undesirable or spoilage agents. Recently, these yeasts have been re-evaluated as a potential biotechnological tool to improve wine composition. Their ability to produce hydrolytic enzymes, glycerol, mannoproteins and other metabolites of oenological relevance allows to enhance the quality of wine [4,5].

A promising approach to reduce wine alcohol content could be to exploit the oxidative fermentative metabolism of some non-*Saccharomyces* yeasts to consume partly the sugar present in the must by respiration rather than fermentation process. As a consequence, the co-inoculation practice of grape must with a selected mixed starter (non-*Saccharomyces/S. cerevisiae*) with partial and controlled aeration of grape juice [6,7] can be considered as one of the best strategies to obtain wine with a reduced content of alcohol and enhanced aromatic quality.

This work is addressed to the characterization of indigenous non-*Saccharomyces* strains by physiological and biochemical analysis, such as resistance to antimicrobial compounds, growth capacity in presence of ethanol and high sugar concentration, β -glucosidase activity, in order to identify the strains suitable for the use as mixed starter for the production of low alcohol wine.

2. Materials and methods

2.1. Yeast strains

Twenty-nine strains of non-*Saccharomyces* species, belonging to the Collection of Fermenting Yeasts of Basilicata University, were used (Table 1). They were isolated from spontaneous fermentation of grapes of

Table 1. Non-*Saccharomyces* strains used in the present study.

Yeast species	Strain code
<i>Hanseniasspora guilliermondii</i>	SNM1 1-1, SNM1 3-2, SNM3 1-1, SNM H, AP 9, TS B, ER 3, TM 4-1, TM 5-1
<i>H. osmophila</i>	ND 1
<i>Torulasporea delbrueckii</i>	425, 365, LC 2-1
<i>Metschnikowia pulcherrima</i>	Mpr 2-49, Mpr1-7, Mpr 2-4, 563, 683, Mpr 1-3, Mpr 2-3, M 1, M 2, M 3, SIA 1, SIA 4
<i>Saccharomyces ludwigii</i>	APG, SIA 2
<i>Pichia fermentans</i>	LM 5-3, SGT 3-1

different origin. The strains were maintained on YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 2% w/v agar).

2.2. Technological characterization

2.2.1. Resistance to SO₂ and CuSO₄, and H₂S production

The resistance to sulphur dioxide (SO₂) was tested by evaluating the growth of non-*Saccharomyces* yeasts on pasteurized agarized grape must added with increasing doses of SO₂ (0, 25, 50, 100, 125, 150, 175, 200 mg/L), whereas the copper resistance was evaluated on agarized Yeast Nitrogen Base (YNB) without amino acids, added with increasing concentrations of CuSO₄ (0, 50, 100, 200, 300, 400, 500 µM). The yeast strains were spot-inoculated on the medium (concentration of about 10⁶ cell/ml) and yeast growth was evaluated after 48 hours at 26 °C.

The ability of the strains to produce different amounts of hydrogen sulphide (H₂S) was tested on bismuth-containing indicator medium BIGGY agar. The medium was spot-inoculated, and plates incubated at 25 °C per 48 hours. The production of H₂S was evaluated in function of yeast spot browning by an arbitrary scale from 0 (white color = no production), 1 (hazelnut = low production), 2 (brown = significant production) 3 (coffee = high production).

2.2.2. Growth in Ethanol and high sugars concentration

The growth test in ethanol was carried out in microplates following the method reported by Eglezos et al. [9] with some changes. As medium, it was used YNB with amino acids, supplemented with a sterile glucose solution (20 g/l), added with different ethanol amounts to obtain final concentrations of 0, 8, 12, 14% (v/v).

The same procedure was used to evaluate the growth in presence of high sugar concentrations, adding to the substrate (YNB) increasing amounts of glucose and fructose, in the same ratio, to reach the final concentrations of 2, 20, 40%.

Yeast cells (about 10⁶ cell/ml) were inoculated in the medium and the microplates were incubated at 26 °C (two days for ethanol test and three days for sugar test). The optical density was measured at 630 nm and the cell growth

was calculated as a ratio (%) between the strain growth in the medium with and without addition of ethanol or sugars.

2.2.3. β-glucosidase activity

This enzymatic activity was evaluated both by qualitative and quantitative methods.

The qualitative β-glucosidase activity was determined on a synthetic medium containing 0.67% YNB with amino acids, 0.5% arbutin, added with 4 ml of ferric ammonium citrate and 2% agar. The strains were spot-inoculated, and the plates incubated at 25 °C for 5 days. The presence of β-glucosidase activity was related to the browning of strain colony.

The quantitative β-glucosidase activity was measured following the method previously described Manzanares et al. [11], with minor changes. Yeasts strains were grown in 25 ml of YNB broth (without ammonium sulphate and amino acids) added with glucose (2%) and ferric ammonium citrate (1%). After incubation at 26 °C for 24 hours in an orbital shaker, cell suspension (10⁶ cell/ml) was centrifuged (3000 rpm for 10 minutes) and 0.2 ml of supernatant was mixed with 0.2 ml of p-nitrophenyl-β-D-glucoside (pNPG). The samples were incubated at 30 °C for 1 hour and the reaction was stopped by adding 1.2 ml of sodium carbonate. The amount of p-nitrophenol released in the reaction was measured spectrophotometrically at 400 nm and the enzymatic activity was expressed as nmol PNP/mL*h comparing to a calibration line of 4-p-nitrophenol solution.

2.2.4. Oxidative stress tolerance

The tolerance to oxidative stress was tested by evaluating strain growth in agarized YPD medium, added with different concentration of H₂O₂ (25, 50, 100, 250 mM), following the protocol reported by Mestre et al. [12]. The different level of strain sensitivity to oxidative stress was correlated to diameter dimension (mm) of the inhibition zone in correspondence of the highest concentration of H₂O₂ tested (250 mM).

2.3. Statistical analysis

All data of the technological parameters were converted into non-dimensional values, assigning the values reported in Table 2.

These values were submitted to cluster analysis, using Ward's method with Euclidean distance by using the statistical package PAST software ver. 1.90 [13].

3. Results

3.1. Technological characterization

3.1.1. Resistance to SO₂ e CuSO₄

All the twenty-nine non-*Saccharomyces* strains exhibited the ability to grow in presence of 100 mg/L of SO₂. Significant variability was detected among the strains, even within the same species (Fig. 1). In general, *T. delbrueckii* strains exhibited the highest resistance; in fact, two strains tolerated 200 and one strain 300 mg/L of SO₂ (the highest tolerance level found among the strains). The Major variability was recorded within the species *H. guilliermondii*, with strain resistance ranging from 100

Table 2. Adimensional values assigned to technological parameters.

Parameters	Values			
	0	1	2	3
SO ₂ resistance ^a	50	100–125	150–175	200
CuSO ₄ resistance ^b	<100	100	200	300
H ₂ S production	no	low	medium	high
β-glucosidase; EtOH /high sugar	V < M-SD	M-SD < V ≤ M	M < V ≤ M + SD	V > M + SD
Oxidative stress ^c	> 10	8–10	5–7	0–4

a = reported as mg/L; b = reported as μM; c = reported as diameter dimension (mm) of inhibition zone.

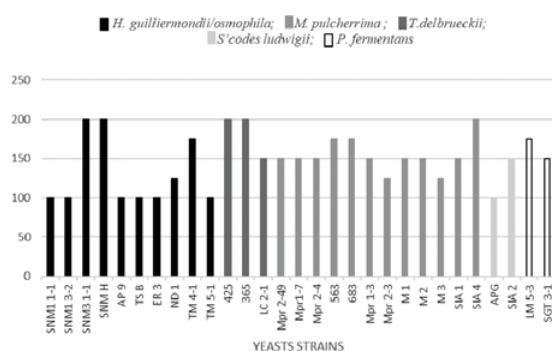


Figure 1. Tolerance level to SO₂.

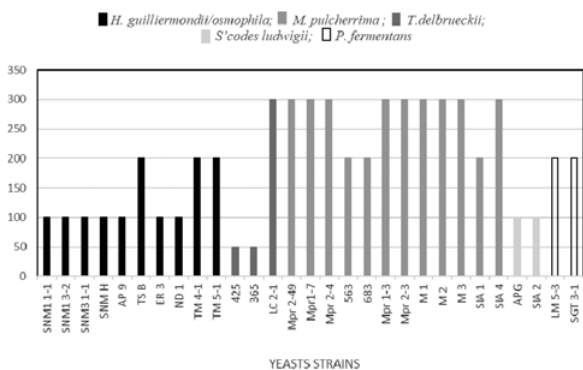


Figure 2. Tolerance level to CuSO₄.

to 200 ppm of SO₂, and *M. pulcherrima* strains, which tolerated SO₂ concentrations ranging between 125 and 200 mg/L.

As regards the copper resistance, the 29 non-*Saccharomyces* strains tolerated concentration of CuSO₄ between 100 and 300 μM and all the strains did not grow in presence of concentrations higher than 300 ppm of CuSO₄ (Fig. 2).

Generally, *M. pulcherrima* strains were more tolerant than *Hanseniaspora* strains; in fact, *M. pulcherrima* grew in presence of 200 and 300 mM of CuSO₄, with 9 strains (Mpr 2–49, Mpr 1–7, Mpr 2–4, Mpr 1–3, Mpr 2–3, M1, M2, M3, SIA 4) exhibiting the highest resistance to the compound. All strains did not grow in presence of concentrations higher than 300 ppm of CuSO₄. Conversely, *Hanseniaspora* strains tolerated copper content ranging from 100 to 200 mM. Low copper tolerance was exhibited also from two *Torulasporea* and the *S'codes ludwigii* strains

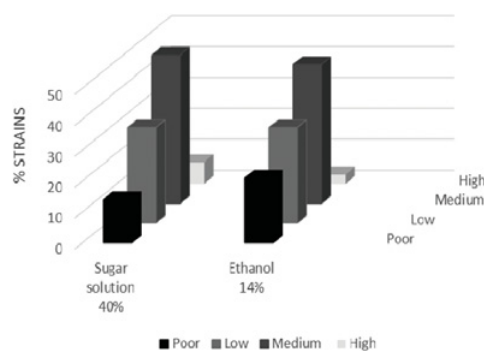


Figure 3. Strain growth in high sugar (40%) and ethanol (14%) concentrations.

3.2. Qualitative production of H₂S

The test for the evaluation of qualitative production of H₂S demonstrated that 79% of yeasts exhibited low production of the compound (hazelnut colonies), mainly strains of *M. pulcherrima* and *H. guilliermondii*, and 21% of strains showed a medium production (brown colour of colonies), exhibited by strains of *P. fermentans*, *T. delbrueckii*, *H. osmophila*, *S'codes ludwigii*.

3.2.1. Growth in ethanol and high sugar concentrations

As regards the test addressed to evaluate the strain tolerance toward high sugar and ethanol concentration, the strains exhibited significant differences among them only in presence of the highest doses of the tested compounds, (14% v/v and 40% for ethanol and sugar, respectively). The strains grew at similar levels for the other doses, both in ethanol and sugar growth test.

About half of the strains exhibited a good cell growth in 14% ethanol (medium growth by 13 strains and high by 1 strain) (Fig. 3).

Similar results were found for growth in high sugar concentrations, 14 strains exhibited medium growth and 2 strains high growth (Fig. 3).

3.2.2. β-glucosidase activity

As regards the qualitative assay of the enzymatic activity, six strains gave negative results (SIA1, SIA 4, 365, LC 2–1, STG 3–1, LM 5–3). Therefore, the quantitative test for β-glucosidase activity was carried out on the 23 strains, resulted positive for the qualitative test.

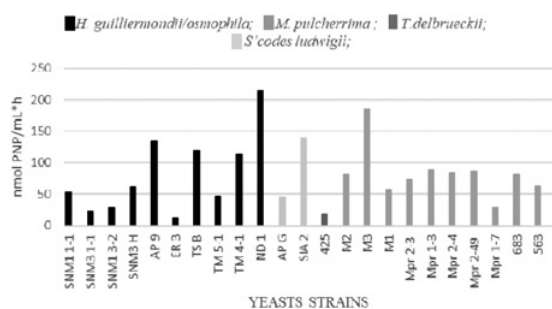


Figure 4. β -glucosidase activity exhibited by 23 strains.

Table 3. Tolerance to oxidative stress of 29 non-*Saccharomyces* strains.

Species	Stress tolerance		
	Low	Medium	High
<i>H. guilliermondii</i> (9)	6	3	—
<i>H. osmophila</i> (1)	—	1	—
<i>T. delbrueckii</i> (3)	3	—	—
<i>S'codes ludwigii</i> (2)	—	2	—
<i>P. fermentans</i> (2)	1	1	—
<i>M. pulcherrima</i> (12)	—	2	10

The results, expressed as nmol PNP/mL*h (Fig. 4), demonstrated that the strains showed a different level of β -glucosidase activity. Some strains exhibited a high enzymatic activity, three strains of *H. guilliermondii* (AP-9, TS-B, TM 4-1), the *S'codes ludwigii* SIA 2, *M. pulcherrima* M3 and *H. osmophila* ND1. These six strains could potentially be used in mixed fermentation to improve the flavor of wines.

3.2.3. Oxidative stress tolerance

The strain tolerance to oxidative stress was evaluated by testing different concentrations of H₂O₂. However, only at the highest dose tested (250 mM) a variability in strain response was found, whereas the concentrations lower than 250 mM did not affected strain growth.

The table reports the results about the oxidative tolerance test obtained by non-*Saccharomyces* species at the higher tested concentration of H₂O₂.

The results (Table 3) show that the strains belonging to *M. pulcherrima* exhibited the highest tolerance to the compound, while the strains of the other tested species presented a low-medium tolerance to 250 mM of H₂O₂.

3.2.4. Statistical elaboration of results from technological characterization

The data obtained by all the tests were converted in adimensional values and the obtained matrix was submitted to cluster analysis in order to differentiate the non-*Saccharomyces* strains. Figure 5 reports Ward's method hierarchical clustering. The dendrogram clearly subdivided the strains in two main groups (a, b).

The group "a" includes strains belonging to the *H. guilliermondii* and *T. delbrueckii* species, whereas the other strains were distributed in the group "b". The principal factors discriminating the two main groups are the ability to grow in presence of high sugar concentration,

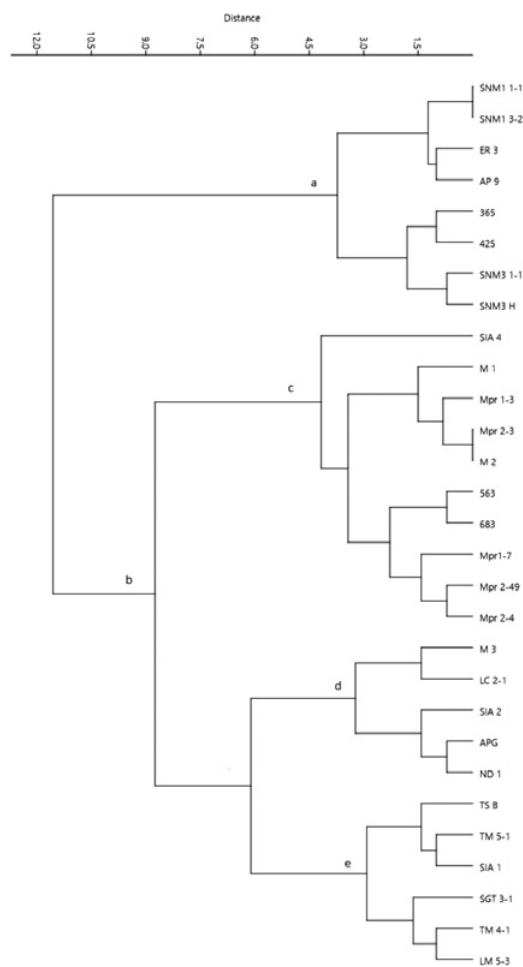


Figure 5. Dendrogram obtained after cluster analysis on data of technological characterization of 23 non-*Saccharomyces* strains.

copper resistance and tolerance to oxidative stress. In fact, the group "a" includes strains exhibiting these traits at the lowest level, whereas strains grouped in "b" were characterized by medium-high ability to grow in presence of high sugar concentration and medium-high tolerance to copper sulphate and oxidative stress.

The ability to tolerate H₂O₂ was more exhibited by the group "b", mainly by the strains of *M. pulcherrima*.

The group "b" can be divided in three subgroups, "c", "d" and "e". The subgroup "c" is the only group composed by strains belonging to the same species, that is *M. pulcherrima*. The characteristics differentiating this cluster are the highest level of tolerance to H₂O₂ and the lowest tolerance to high ethanol concentration.

The other subgroups are composed by strains belonging to different species, such as *P. fermentans*, *H. guilliermondii*, *H. osmophila*, *S'codes ludwigii*, two *M. pulcherrima* strains and one *T. delbrueckii* strain. The characteristic mainly differentiating "d" from "e" subgroup is the β -glycosidase activity, which was low/very low in "d" and medium/high in "e". Furthermore, the subgroup "d" includes the strains showing the best combination of technological parameters tested. In fact, the strains grouped in "d" were characterized by medium/high ability to tolerate high concentration of sugar and ethanol, medium/high β -glycosidase activity and medium level of tolerance to oxidative stress.

4. Conclusions

This preliminary screening of indigenous non-*Saccharomyces* yeasts might be an useful tool to individuate some strains characterized by traits of oenological interest and potential candidates in pure or mixed starter cultures for the production of low alcohol wine. Our results confirm that non-*Saccharomyces* yeasts, previously considered as spoilage microorganisms, can be considered as an interesting source of biodiversity, with positive applications to grape-must fermentation.

In particular, *H. osmophila* and *S'codes ludwigii* exhibited interesting and desirable properties to improve wine sensory profile, such as the highest β -glucosidase activity and the good resistance to osmotic stress, being able to survive in fermentation must condition.

Also *M. pulcherrima* strains showed interesting technological traits, but, due to its sensibility to high concentrations of ethanol, it could be used only in mixed culture with *S. cerevisiae* in order to complete the fermentation process. In particular, all *M. pulcherrima* strains exhibited a high tolerance to hydrogen peroxide, that is one of reactive oxygen species produced by the sugar respiratory catabolism, which is potentially toxic to yeast cell. Non-*Saccharomyces* strains able to tolerate H₂O₂ are suitable to be used in the first stage of fermentation process, under aerobic controlled condition, in order to oxidize sugars present in the grape must and consequently to reduce ethanol production.

In conclusion, mixed starter cultures with non-*Saccharomyces* strains, carefully selected in function of wine characteristics and market trends, can be considered an innovative biotechnological tool not only to improve wine quality complexity, but also to satisfy the current

challenge of wine industry addressed to “lower alcohol wines”.

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