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# Distribution and correlation of three oenological traits in *Saccharomyces cerevisiae*

Laura CORTE, Paolo RELLINI, Francesco SCIASCIA, Raffaele DE NICOLA, Fabrizio FATICHENTI, Gianluigi CARDINALI*

Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali e Zootecniche, Sezione Microbiologia Agroalimentare e Ambientale, Università degli Studi di Perugia, Borgo 20 Giugno 74, 06121 Perugia, Italy

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**Abstract -** The improvement of yeast starters for wine making, with classical genetic techniques, relays on the effective independence of the main oenological traits in order to combine them optimally. The analysis of three characters (ethanol production, volatile acidity and fermenting rate) in 787 *Saccharomyces cerevisiae* strains, isolated from several parts of the world and substrates, shows moderate correlation between the volatile acidity and the other two traits, whereas ethanol production and fermenting rate appear more correlated. The minimum number of strains necessary to start selection or genetic improvement programs is discussed. The correlation between the oenological traits and the source of isolation showed that the best strains can be obtained from wineries and wine, whereas the isolates from grapes and must are less performing on the average.

**Key words**: yeast, *Saccharomyces,* physiology, character independence, oenology.

## INTRODUCTION

The yeast starters for the oenological industry have been originally selected from wine and related substrates all over the world (Castelli, 1948, 1964; Minárik, 1965; Parle and Di Menna, 1965; Castelli and Georgantas, 1970; Rosini, 1983; Mora *et al.,* 1988; Martini, 1993; Miklos *et al.,* 1994; Vaughan-Martini and Martini, 1995; Maifreni *et al.* 1999; Sipiczki *et al.,* 2001; Fleet *et al.,* 2002; Vilanova and Massneuf-Pomarede, 2005). This search is now hampered by a strong reduction of the biodiversity and variability available caused by the massive use of selected strains, mostly commercialised as active dried yeast. This situation and the increasing requests for new and typical strains by the oenological industry suggest the necessity of some system of genetic improvement of the technological traits (Romano *et al.,* 1985; Volschenk *et al.,* 1997; Ramirez *et al.* 1999; Pretorius, 2000; Rainieri and Pretorius, 2000). However, the well known hostility of most consumers toward genetic engineered organisms discouraged the use of molecular cloning even in the countries where the use of such organisms is not strictly forbidden. These circumstances are leading to the paradox of a request for new improved starters necessary to meet the expectations of an increasing refined winery market, which cannot be fulfilled. For these reasons, there is an increasing interest to develop new procedures based on classical genetics strategies (Marullo *et al.,* 2004). This new trend has to take into consideration some peculiarities of the yeast *Saccharomyces cerevisiae* such as the diffuse homotallism, the multi-locus encoding of the quantitative traits of oenological interest (Mortimer *et al.,* 1994) and the necessity to consider always more characters to match the increasingly demanding expectations of the oenologists (Rainieri and Pretorius, 2000). In this picture, the distribution and the effective independence of the technological traits is critical for the success of the programs for the genetic improvement of the starters.

In this paper we have analysed 787 *S. cerevisiae* strains isolated in different parts of the world and from diverse substrates in order to describe the distribution and relation among three characters of oenological interest such as ethanol production (fermenting power), production of carbon dioxide in the first five days (fermentation rate) and the volatile acidity developed in the course of the fermentation.

## MATERIALS AND METHODS

**Strains.** The technological data of the 787 *Saccharomyces cerevisiae* strains considered were retrieved from the databank of the DBVPG (Dipartimento di Biologia Vegetale di Perugia) Industrial Yeasts Collection where are collected well over one thousand strains isolated from different substrates and from several regions of the world. These 787 isolates were chosen for the availability of the data concerning all the three oenological traits under study, obtained with the methods reported below (Rosini *et al.,* 1979).

* Corresponding author. Phone: +39 075 585 6478;
Fax: +39 075 585 6470; E-mail: gianlu@unipg.it

**Ethanol production (EP).** Müller valve-tapped Erlmeyer flask with 70 ml of sterile synthetic must (1 l solution: 1 g $KH_2PO_4$, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 3 g yeast extract, 27% w/v glucose, 4 g of tartaric acid and 2 g hydrogen potassium tartrate) were inoculated with yeast pre-cultures and weight before incubation at 25 °C. During the fermentation the samples were weighted everyday until the weigh remained constant. The $CO_2$ loss was evaluated to calculate the ethanol content according to the formula *EP= kC*, where k is 1.78 and C indicates the $CO_2$ loss in grams. (Ciani and Rosini, 1987). These values were controlled and confirmed by ebulliometric analysis (i.e. based on the different boiling point of solutions with divers alcohol concentrations). It is important to highlight here that the interest of this study is in comparing character intensities of several strains, more than dealing with extremely precise determinations, which can be better obtained with other methods in larger scale fermentations, unaffordable when handling several strains simultaneously.

**Fermentation rate (FR) and volatile acidity (VA).** The procedure to obtain FR its the same used to obtain EP with the only difference that 68 ml of sterile synthetic must were inoculated with 2 ml of culture obtained from a 24 h saturated pre-culture. The difference of weight observed in the first 5 days gave us the fermentation rate and are expressed directly as g $CO_2$/70 ml.

The determination of the VA was carried out by titration at the end of fermentation using 5 ml wine distilled in steam flow.

**Statistical analyses.** Technological data were retrieved from the "*Industrial Yeasts Collection DBVPG"* at the University of Perugia (http://www.agr.unipg.it/dbvpg/home.html). Statistical analyses were carried out with the Microsoft Excel application and with Kaleida Graph (http://www.synergy.com/pricing.htm). The Kolmagorov-Smirnov normality test was carried out with the Le Progiciel package (http://www.fas.umontreal.ca/BIOL/Casgrain/en/index.html). The ternary plot graph was obtained with the trial version of JMP 6 (SAS Institute). Spearman rank correlations were calculated according to two different procedures (Legendre and Legendre, 1998) finding correspondence among the values.

## RESULTS AND DISCUSSION

### Distribution of the oenological characters

Data classification of the three characters under study shows that the ethanol production (EP) has a range from 6 to 16% v/v with mean, mode and median respectively 11.30% v/v, 10.85% v/v, 11.39% v/v (Fig. 1A). Similarly, the volatile acidity (VA) (Fig. 1B) has the three statistical descriptors clustered between 1.08 and 1.10 g/l (mean = 1.1 g/l, mode = 1.08 g/l, median = 1.10 g/l). Conversely, the fermentation rate (FR) shows an asymmetrical distribution with the majority of the strains ranging from 0 to 1 g $CO_2$/70 ml (corresponding to 0 to 1.8% v/v ethanol) and a few with FR up to 4 g $CO_2$/70 ml (Fig. 1C). This asymmetry is well evaluated by the difference between mean, mode and median, 0.81, 0.76 and 0.71 respectively. The Kolmagorov-Smirnov normality test indicated that no character has a normal distribution with the exception of VA at 0.01 level of significance of the null hypothesis.

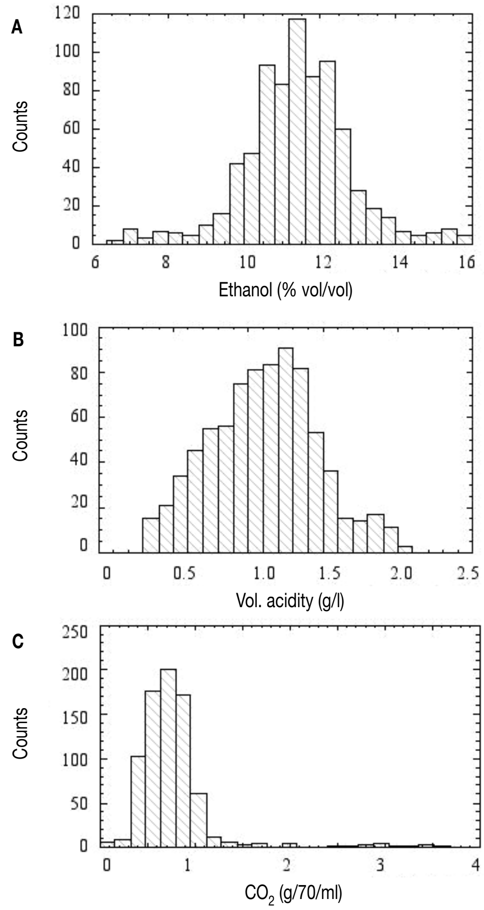


FIG. 1 – Distribution histograms showing the counts of classified values of the three characters under study. Panel A, ethanol production; panel B, volatile acidity; panel C, fermentation rate.

These data show that only few strains are able to produce more than 1.8% v/v ethanol within 5 days in the experimental conditions described in the Materials and Methods section. The 93 strains with FR values over 1 g $CO_2$/70 ml represent an important reservoir of variability to take advantage of this trait, which is extremely important because a fast production of ethanol tends to decrease the risks inherent with the growth of apiculate yeast during the first days of the wine fermentation.

### Correlations among oenological traits

The main aim of this communication is to evaluate, in a large number of *S. cerevisiae* strains, whether three oenological characters show enough independence among themselves in order to employ classical genetic techniques to produce new improved cultures.

The analysis of these data, according to the Spearman rank correlation, shows negative values between the VA and the other two traits (-0.258 VA vs. EP, -0.503 VA vs. FR). Particularly interesting is the low correlation between VA and EP, suggesting that it is largely possible to obtain yeast strains coupling high fermentation purity (low concentrations of acetic acid) and high EP, which represent the minimum prerequisite for a good wine starter. The correlation between EP and FR is positive although not high (0.529), indicating that high ethanol producer tend to have high FR, with still large

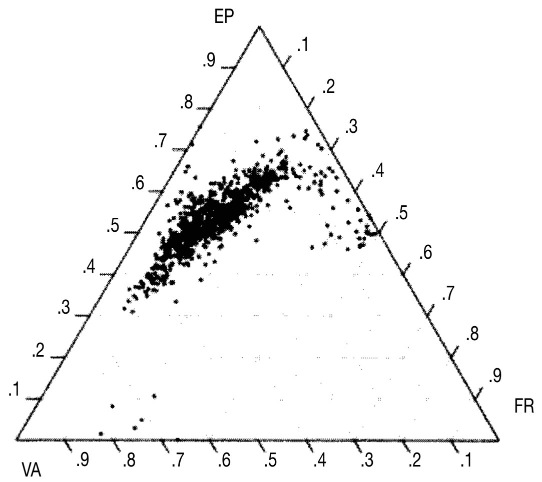


FIG. 2 – Triplot showing the strain distribution according to the three traits under study. Values were normalized before the plotting according to the transformation y′ =(y-min)/(max-min), were y and y′ represent the value before and after the normalization, min and max indicate the minimum and maximum of the distribution.

TABLE 1 – Spearman Rank Correlations among oenological traits in samples of different size

| Sample size* | EP-VA | FR-VA | EP-FR |
|---|---|---|---|
| 787 | -0.258 | -0.503 | 0.529 |
| 400 | -0.250±0.016 | -0.502±0.013 | 0.536±0.009 |
| 200 | -0.305±0.029 | -0.535±0.026 | 0.538±0.019 |
| 100 | -0.228±0.048 | -0.496±0.028 | 0.513±0.034 |
| 50 | -0.286±0.071 | -0.465±0.042 | 0.542±0.039 |

* The first sample (787 strains) is the general set with all strains considered. All other sets were obtained by random sampling of the general set. Each correlation value represents the average of twenty random resamplings. 95% confidence intervals are reported.

spaces for further improvements. The same correlations were carried out with 21 strains recently isolated from quality wines, finding that this subset is not significantly different from the whole dataset in the correlations between VA vs. EP (-0.270), whereas the correlation EP vs. FR is much higher (0.774) and the FR vs. VA correlation is much weaker (-0.292). These figure suggest that selectors have traditionally tried to choose strains with the ability to produce much ethanol trying to keep the VA low, but paying less attention to the FR. The ternary plot (Fig. 2) gives a synthetic view of the correlation among traits and of the strain distribution, showing that the vast majority of the strains have high VA, medium EP and low FR, which represent the worst possible combination in oenology. The few strains with low VA, high EP and reasonably high FR values, represent an interesting starting point for breeding programs and for direct use as starters. The ternary plot confirms that the variability of the FR is moderate, raising the question on the reason on whether this is due to a scarce selection or to some reason inherent to the yeast metabolism. Whatever the answer, the selection of wine starters with improved FR seems to represent a challenging and promising activity.

**Character correlation in smaller sets of strains**

These correlations were obtained by analysing a large set of strains, raising the question on whether smaller sets would maintain the same levels of trait independence or, in other terms, how small can a set be in order to find strains with good levels of character independence, considering that small samples increase the bias of this estimator (Zimmerman *et al.*, 2003). Subsets of 400, 200, 100 and 50 strains were obtained by random resampling with an Excel macro written for this scope, using a procedure already tested with success (Corte *et al.,* 2005). For each subset twenty independent resamplings were carried out in order to calculate the three pair-wise characters rank correlations and, finally, their mean and the 95% confidence.

The analysis of the mean correlations in the 4 different subsets (Table 1) shows that the correlation EP vs. VA decreases to -0,305 in the 200 strains set and grows to -0,228 in the 100 strains set. Similarly, the correlation FR vs VA decreases from -0.503 of the general set to -0.535 in the 200 strains set and increases to -0.465 with 50 strains. Conversely, the EP vs. FR correlations show little if any change between the resamplings and the original dataset. The mean of the confidence intervals increase from 0.13 with 400 strains to 0.51 with 50 strains.

The variations due to the random resamplings can be evaluated by considering the maximum variation between the mean values of each pair-wise correlation and the correlation value obtained with the full dataset, finding that the EP-VA correlations have a maximum variation of 29%, whereas the FR-VA and EP-FR correlations vary no more than 8% and 6%, respectively. Since the maximum variations can be found when 100 strains were resampled, this analysis suggests that no less than 200 strains should be considered in selection programs in order to have enough variability and character independence.

In order to further investigate on the frequencies of strains with the best oenological performances, we have calculated for each pair of traits the number of strains (counts) presenting different combinations of character intensities. In figure 3A are shown the counts (y axis) of strains with EP equal or higher than the value indicated in the z axis and with AV minor or equal than the figures reported in the x axis. Similarly, in the other two panels are reported the counts of strains according to EP vs. FR (Fig. 3B) and to VA vs. FR (Fig. 3C). The first histograms (Fig. 3A) shows that the counts decrease more at the decreasing of the VA than at the increase of the EP and the smallest count (6 strains) corresponds to 15% v/v ethanol and 0.4 g/l VA. In general, the counts of strains with VA ≤ 0.4 are low and do not increase significantly at any ethanol concentration. Similarly, the counts in figure 3B decrease very steeply at the increase of the FR, more than at the increase of EP. Finally, the counts in figure 3C show a very steep decrease both in the VA and FR direction. The least count is 1 strain with AV 0.4 g/l and FR 3.5 g/70 ml.

All together these data show that the simple selection of strains from the wineries or from the environment can optimise the strain performance for two characters, but generally failed to optimise all the three traits considered in this study. These findings suggest at least three preliminary conclusions. Firstly, a large consensus on the traits really important should be reached among microbiologists and oenologists in order to better direct the selection and the genetic

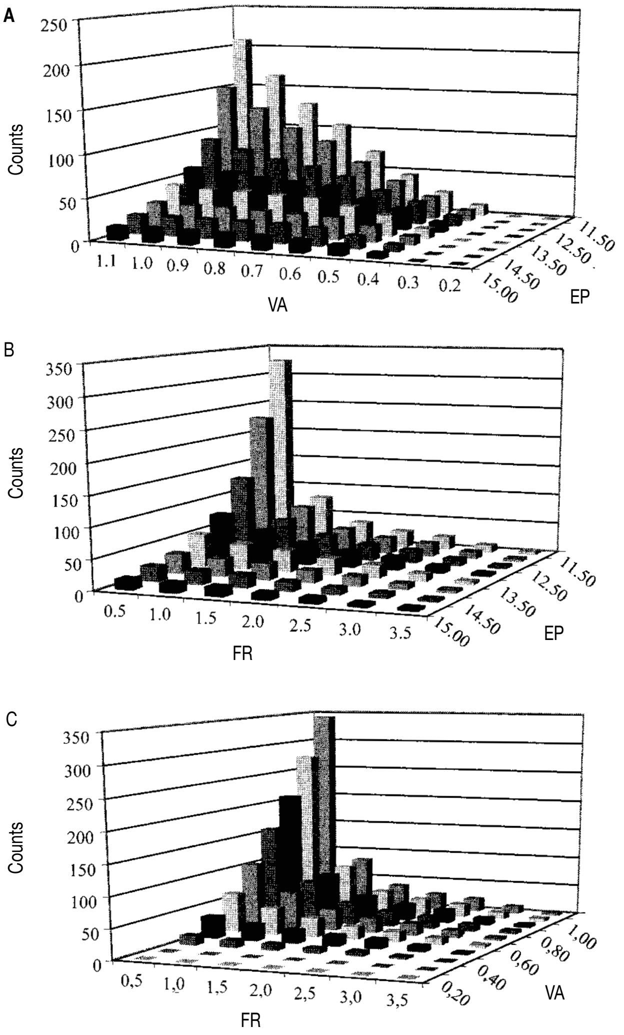


FIG. 3 – Tridimentional histograms representing the distribution of the oenologically more interesting strains according to the three pairs of traits. Panel A, volatile acidity (VA) vs. ethanol production (EP); panel B, EP vs. fermentation rate (FR); panel C, VA vs. FR.

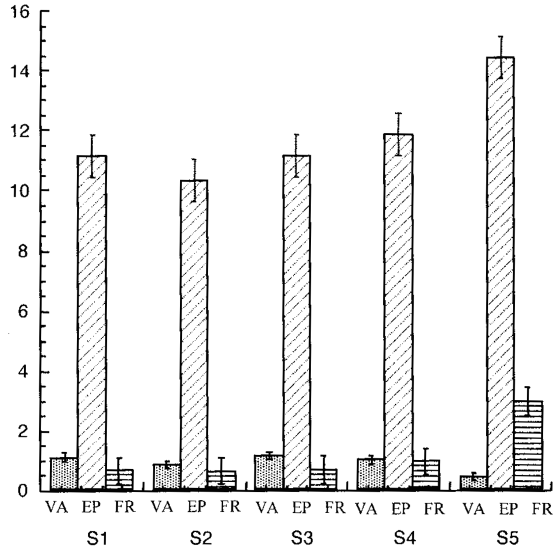


FIG. 4 – Distribution of the three oenological traits under study according to the isolation source of the yeasts. S1, sugar unrelated substrates (e.g. water, soil etc.); S2, fruit surfaces, S3, must and sweet juices; S4, wine; S5, winery surfaces and equipment. Figures reported on the y-axis are expressed as: ethanol production (EP), % v/v; volatile acidity (VA), mg/l; fermentation rate (FP), g $CO_2$/70 ml.

improvement of new starters. Secondly, a comprehensive scoring system should be developed in order to define strains with a single synthetic parameter. Obviously, the relative weight of each character needs to be evaluated with great care in order to avoid that this conventional parameter becomes exceedingly subjective. Finally, these data analyses show that there is enough variability and character independence to consider the possibility of genetic improvement programs to produce strains optimised for the traits necessary to fulfil the requirements of modern oenology.

**Influence of the isolation source**

Since most of the strains were isolated from wine related environments, the relative scarcity of good wine strains poses a question on the importance of the isolation source. We have therefore grouped the 787 strains into five classes according to their isolation source as follows. S1 includes all sugar-free sources as water, soil wounds etc., the S2 class is represented by grape and other fruit surfaces. In S3 and S4 are clustered all strains isolated respectively from grape must and wine. Finally, in S5 there are the strains isolated from winery surfaces and equipment. The distribution of the oenological traits through these five classes in figure 4 shows that the best strains (high EP and FR, low VA) were isolated from the wineries; although these strains are relatively few in comparison to those isolated from wine, must and grapes to allow a strong conclusion, these data confirm that the winery is a sort of privileged selective place as proposed by Martini and co-workers (Martini, 1993; Martini and Ciani, 1996). Interestingly, there are 11 strains with maximum level of EP (15.96) in the S4 class and 5 in the S5, suggesting that excellent strains can be isolated both from wine and wineries. Interestingly, the frequencies of strains with high EP (Table 2) show that no remarkable difference exists between the first three sources of isolation, meaning that high alcohol content, rather than the presence of sugar, is the primary force to select good starters.

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TABLE 2 – Frequency of yeast strains according to their EP value and to the origin of isolation

| EP (%) | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| >15 | 0.0 | 0.0 | 0.2 | 7.7 | 27.8 |
| >14 | 0.0 | 1.9 | 0.4 | 12.7 | 61.1 |
| >13 | 3.4 | 5.7 | 4.6 | 21.8 | 88.9 |
| >12 | 27.1 | 26.4 | 27.2 | 38.7 | 83.3 |

S1: substrates without sugar, S2: grapes and fruits, S3: must, S4: wine, S5: wineries and equipment.

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