# *Saccharomyces cerevisiae* yeast strains from the Linosa Island

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## ABSTRACT

We report on the *Saccharomyces* yeasts of oenological interest found on grapes of the Linosa Island. This is a small island in the Mediterranean sea (about 160 km away from Sicily or Tunisia), where no wineries are present and no commercial wine yeast strains were ever used.
In 2008 two vineyard (both with grapes of the "Muscat of Alessandria" *cv*) were established. Some scattered plants (Inzolia *cv*) were also present ("old vines"). In 2009 samples from the new vineyards and from the old vines were collected during maturation, to assess the biodiversity of yeast species present on the grapes. No *Saccharomyces* were found. Grapes from the new vineyards were harvested and left to dry in the sun and the yeasts species present after five days were analyzed. 53 *Saccharomyces* isolates were retrieved. All the isolates were ascribed to the *S. cerevisiae* species and distinguished in four strains by way of mt-DNA RFLP analysis. A phenotypic characterization of one representative per each strain was performed.

## RIASSUNTO

In questo lavoro descriviamo i lieviti *Saccharomyces* di interesse enologico trovati sulle uve dell' Isola di Linosa. Si tratta di una piccola isola del Mediterraneo (circa 160 km di distanza dalla Sicilia e dalla Tunisia), in cui non sono presenti cantine e non sono mai stati usati ceppi di lievito commerciali. Nel 2008 sono stati istituiti due vigneti (entrambi della *cultivar* "Moscato d'Alessandria" ). Sull'isola erano presenti alcune piante sparse (*cultivar* Inzolia, qui chiamate "viti vecchie"). Nel 2009, nel corso della maturazione, sono stati prelevati dei campioni dai vigneti nuovi e dalle viti vecchie per valutare la biodiversità delle specie di lieviti presenti sulle uve. Non è stato ritrovato nessun isolato di *Saccharomyces*. Le uve provenienti dai vigneti nuovi sono state raccolte e lasciate essiccare al sole e sono state analizzate le specie di lieviti presenti dopo cinque giorni. In questo modo sono stati isolati 53 rappresentanti della specie *Saccharomyces cerevisiae*, distinti in quattro ceppi grazie ad analisi RFLP del mt-DNA. È stata eseguita una caratterizzazione fenotipica di un rappresentante per ogni ceppo.

## INTRODUCTION

In recent years the taste of consumers has privileged novelty, with an interest sometimes directed toward products which relate to particular regions of the world. Therefore a research on the enological potential of "unusual" yeast species has been stimulated, with the attention been put on non-*Saccharomyces* yeasts (e.g. (Ciani et al., 2010)) or *Saccharomyces* yeasts from specific areas (Di Maio et al., 2012), being these different from the commercial yeast strains commonly adopted in the winery. This however has been made more difficult by the

ever increasing diffusion of industrial practices with the use of commercial yeasts, given the ease with which these strains install themselves in the wineries and with which they move between these and the vineyards (discussed in Bisson, 2012; Mendez-Vilas et al., 2010; Valero et al., 2005). Therefore an ideal situation for the discovery of new yeasts species of potential interest for enology, was that provided by Linosa, a small island in the Mediterranean Sea where no wineries were present and the only commercial *S. cerevisiae* yeasts in use were those for bread-making. No large vineyards were present on the island until 2008, when two experimental fields were established under the sponsorship of the IRVO. Some scattered Inzolia plants were however present, to the use of the local families ("old vineyards").

Here we report on the biodiversity of the yeast species recovered from the grapes of the newly established fields and from the old vineyard, focusing on the *Saccharomyces cerevisiae* strains that were found.

## MATERIALS AND METHODS

*Yeast strains*

The *S. cerevisiae* L404 and 6167 strains and the *S. bayanus* strain 11719 belong to the DIPROVAL collection (University of Bologna). The *S. cerevisiae* BA11, ICV-k1, EC1118, ICV-D254 and RC212 are commercialized by Lallemand Inc. (Canada). The *Saccharomyces* strains A1-38 and A1-42 (Di Maio *et al*., 2012) Cpts1 33 and Cpts1 25, belong to the IRVO *Saccharomyces* yeasts collection. The *Metschnikowia pulcherrima* Mp03 strain and the *Hanseniaspora uvarum* Hu03 strain belong to the IRVO non-*Saccharomyces* yeasts collection. The *Saccharomyces cerevisiae* DI yeast strain used for bread-making on the Linosa Island, is distributed by Distillerie Italiane (Lesaffre Italia SPA).

*Sampling sites*

In 2008 two experimental vineyards (1 and 2) were established by the IRVO on the Linosa Island. This is a small island of volcanic origin (35°52'00'' North; 12°52'00'' East) about 160 km away from the coasts of Tunisia and Sicily (Italy). In both cases implanted vines were *alberelli* (shrub-like short trees) of the "Muscat of Alexandria" cultivar. Copper and sulfur were used for phytosanitation and the vines were protected with fences. Some scattered plants were also present on the Island; mostly of the Inzolia cultivar, not arranged in rows; implantation dated to about 30 years ago. No phytosanitation treatments were applied to these old vines, which were not protected and remained accessible to rabbits (quite abundant on the island). Samples from these vines were also taken. In what follows these samples are referred as samples from the "old vines".

*Grape sampling*

During summer of 2009 (from July 9$^{th}$ to September 2$^{nd}$) samples were taken once every two weeks, from vineyard 1, 2 and from the old vines. Five samples were taken from each site. A total of 15 samples, of 200-300 grams each, were collected and the progress of grape maturation was monitored by measuring the $^{o}$Brix at each sampling. Grapes were put in sterile bags, kept cold and transferred to the IRVO laboratory in Palermo within 12 hours. Additionally at ripening, grapes from field 1 and 2 were picked and left to dry in the sun on the island. After five days a sample from these grapes was taken, grapes were pressed and 20 mL of juice were diluted with 20 mL of glycerol. The mixture was stored at -20°C transferred cold to the laboratory in Palermo and stored at -20°C until further analysis (in what follows this kind of sample is called "juice of the sun-dried grapes").

*Fermentations*

To study the yeast species present on the grapes at the end of spontaneous fermentations, samples from field 1, 2 and old vines were weighted and pressed to obtain a homogeneous must; of this, an aliquot was taken for analysis ("early musts"); of what remained 30 mL were poured in 50 mL sterile tubes and were let to spontaneous fermentation (about 15 days at 28$^{o}$C; "fermented musts") until no effervescence could be seen. Residual glucose was then measured using the reagents of the Diabur-Keto-Test® 5000 kit (Roche) following manufacturer's procedures. Fermentations were also performed to obtain a phenotypic characterization of representative isolates of the *S. cerevisiae* yeast strains, found on the Linosa Island (see below).

*Microbiological analyses*

To identify the yeast species present on the grapes, 200 µL of freshly squeezed grapes ("early musts"), or of "fermented musts" (see " *Fermentations*"), or juice from the sun-dried grapes ("sun dried grapes"), were diluted with 0.1% (w/v) peptone sterile distilled water. These were then dispersed on WL Nutrient Agar (Oxoid, Hampshire, UK) plates and on Agar Lysine (Oxoid, Hampshire, UK) plates. Diphenyl was added to slow-down moulds proliferation and plates were incubated at 28°C. After 5 days, plates with 20-250 colonies were analyzed. Yeasts were identified, based on colony morphology (Cavazza and Poznanski, 1998; Cavazza et al., 1992; Pallmann et al., 2001; Romancino et al., 2008) and by microscopic analysis. A significant number of colonies (30-100 per sampling) were purified by streaking on WL Nutrient Agar plates. After 5 days at 28$^{o}$C, 518 Malt Agar (30 g/L Malt extract, 15 g/L Agar; Oxoid) collection tubes were prepared and yeasts slants were stored at 4$^{o}$C.

*Phenotypic characterization*

Fermentative vigor and $SO_2$ tolerance were measured according to (Caridi et al., 2002). The L404 *S. cerevisiae* strain was used as positive control. Not inoculated must was used as negative control.

Fermentative power was measured every day during a time period of 38 days, after adding glucose to the unfermented musts, up to 300g/L of sugars (Caridi et al., 2002). Glucose+fructose, ethanol, volatile acidity, glycerol, malic acid, lactic acid and citric acid were measured by using a Winescan apparatus (FOSS Integrator, Denmark) at 15 days from the start of fementation. A statistical analysis was performed using the analysis of variance (ANOVA) and the least significant difference (LSD) test to determine statistically different values at a significance level of $p < 0.05$.

Killer activity of the isolates was measured as in (Regodón et al., 1997), by plating on Ba11 *S. cerevisiae* yeast cells. The *S. cerevisiae* ICV-k1 and ECC118 strains were used as positive controls; the *S. cerevisiae* ICV-D254 and RC212 strains were used as negative controls.

Production of $H_2S$ was measured according to (Nickerson, 1953). The Cpts1 33, Cpts1 25, A1-38, A1-42 strains were used as references (from a very low to a high production).

*Molecular analyses*

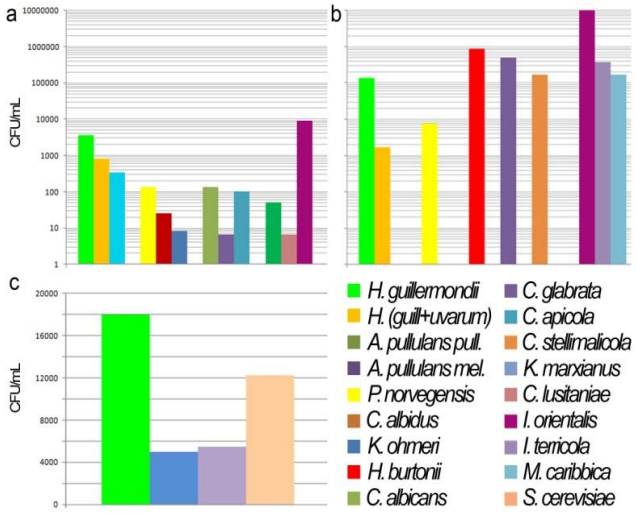
A total of 517 yeasts isolates were analyzed by PCR-RFLP (Granchi et al., 1999). Amplicons were digested with the appropriate restriction enzymes (*HinfI*, *HaeIII*, *DdeI*; New England BioLabs, Hertfordshire, England) to identify polymorphism groups. When necessary one isolate per each group was chosen, the D1/D2 region of the 26S rDNA was amplified, and the DNA was sequenced and analyzed at the DBVPG (Industrial Yeasts Collection,

University of Perugia- Department of Applied Biology) according to (Fell et al., 2000). Species-specific PCR reactions (Sabaté et al., 2000) were performed on 53 isolates of the *Saccharomyces* genus. PCR products were analyzed by agarose gel electrophoresis. The *S. cerevisiae* strain 6167 and the *S. bayanus* strain 11719 were used a positive and negative controls respectively.

## RESULTS AND DISCUSSION

From the sampling of the Linosa grapes (from the old vines as well as the new vineyards), a total of 3939 colonies were isolated and morphology of each colonies was analyzed. 17 different morphologies were identified, and for each a different restriction pattern was found, except for the morphologies corresponding to *Aureobasidium pullulans* var *pullulans* and var *melanogenum* species, which showed the same pattern; also, one morphology was resolved into two different restriction patterns, corresponding to the *H. guilliermondii* and *H. uvarum* species.

The figure on the right, provides an overview of the yeast biodiversity on Linosa. Average CFU/mL values for each of the yeast species is shown. No *S. cerevisiae* isolates were recovered from the vineyard grapes (a, "early musts"; b, "fermented musts"; y axis: log scale 1-$10^7$ CFU/mL).
These were instead retrieved from ripe grapes from the new fields, which were left under the sun for five days (c, "sun-dried grapes"; y axis: linear scale 1-20000 CFU/mL).




The PCR-RFLP patterns of the *Saccharomyces* isolates (a total of 53) was analyzed. Species-specific primers (SC1 and SC2) were used and the isolates were all found to be members of the *S. cerevisiae* species. By *HinfI* digestion of the mt-DNA, these were ascribed to four polymorphism groups: 45 isolates to group 1; 6 isolates to group 2; 1 isolate to group 3; 1 isolate to group 4. Also the RFLP pattern of the bread-making yeast strain ("DI", a *S. cerevisiae* representative), was analyzed. This was found to be different from that of the *S. cerevisiae* yeasts found on the sun-dried grapes.
To characterize these *S. cerevisiae* yeasts, four "sun-dried grapes" isolates and one DI isolate, were assayed for five technological and eight enochemical parameters. Values were compared with those of the commercial L404 strain and of the blank. A statistical analysis (Least Significant Difference) was performed. Results are shown in the Table below (different letters indicate statistically significant differences at $p<0.05$).

| Technological parameters | Group I | Group II | Group III | Group IV | DI | L404 | Blank |
|---|---|---|---|---|---|---|---|
| Fermentaitve vigor 2 days | 4.51 (0,24)$^{c}$ | 3.98 (0.40)$^{bc}$ | 2.72 (0.37)$^{b}$ | 4.95 (0.61)$^{c}$ | 3.88 (1.07)$^{bc}$ | 4.39 (0.47)$^{c}$ | 0.02 (0.00)$^{a}$ |
| Fermentative vigor 7 days | 9.48 (0.17)$^{b}$ | 9.54 (0.11)$^{b}$ | 9.15 (0.55)$^{b}$ | 9.58 (0.31)$^{b}$ | 9.74 (0.03)$^{b}$ | 9.71 (0.47)$^{b}$ | 0.03 (0.01)$^{a}$ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $SO_2$ tolerance | 5.60 | 5.03 | 2.75 | 5.23 | 4.52 | 4.33 | 0.00 |
| 2 days | $(1.39)^{b}$ | $(1.03)^{ab}$ | $(0.27)^{ab}$ | $(0.33)^{b}$ | $(0.88)^{ab}$ | $(1.65)^{ab}$ | $(0.00)^{a}$ |
| $SO_2$ tolerance | 10.03 | 9.63 | 9.72 | 9.93 | 9.84 | 9.47 | 0.02 |
| 7 days | $(0.33)^{b}$ | $(0.04)^{ab}$ | $(0.20)^{ab}$ | $(0.07)^{ab}$ | $(0.20)^{ab}$ | $(0.41)^{ab}$ | $(0.03)^{a}$ |
| Fermentative power | 15.87 | 15.91 | 13.55 | 14.45 | 13.88 | 15.25 | 0.11 |
| (at 38 days) | $(0.10)^{cd}$ | $(0.30)^{d}$ | $(1.65)^{b}$ | $(1,12)^{bcd}$ | $(0.99)^{bc}$ | $(013)^{bcd}$ | $(0.01)^{a}$ |
| **Enochemical paramaters** | **Group I** | **Group II** | **Group III** | **Group IV** | **DI** | **L404** | **Blank** |
| Ethanol | 11.27 | 11.26 | 11.26 | 11.19 $(0.10)^{b}$ | 11.30 | 11.35 | 0.17 |
| (% v/v) | $(0.04)^{bc}$ | $(0.07)^{bc}$ | $(0.02)^{bc}$ | | $(0.10)^{bc}$ | $(0.03)^{c}$ | $(0.01)^{a}$ |
| Volatile acidity | 0.68 | 0.56 | 0.56 | 0.75 | 0.38 | 0.54 | 0.13 |
| (g/L) | $(0.02)^{d}$ | $(0.00)^{c}$ | $(0.01)^{c}$ | $(0.02)^{e}$ | $(0.01)^{b}$ | $(0.00)^{c}$ | $(0.01)^{a}$ |
| Malic acid | 0.94 | 0.77 | 0.92 | 1.13 | 0.82 | 1.05 | 1.62 |
| (g/L) | $(0.05)^{b}$ | $(0.01)^{a}$ | $(0.01)^{b}$ | $(0.04)^{c}$ | $(0.06)^{a}$ | $(0.01)^{c}$ | $(0.01)^{d}$ |
| Citric acid | 0.33 | 0.32 | 0.35 | 0.30 | 0.45 | 0.37 | 0.42 |
| (g/L) | $(0.01)^{ab}$ | $(0.00)^{ab}$ | $(0.01)^{bc}$ | $(0.03)^{a}$ | $(0.01)^{e}$ | $(0.01)^{c}$ | $(0.01)^{d}$ |
| Total acidity | 5.20 | 4.80 | 5.05 | 5.60 | 4.95 | 5.50 | 3.97 |
| (g/L) | $(0.00)^{d}$ | $(0.00)^{b}$ | $(0.07)^{cd}$ | $(0.14)^{e}$ | $(0.21)^{cb}$ | $(0.02)^{e}$ | $(0.01)^{a}$ |
| Gluc+Fruct | 2.30 | 2.33 | 2.38 | 2.37 | 2.31 | 2.80 | 191.67 |
| (g/L) | $(0.01)^{a}$ | $(0.12)^{a}$ | $(0.03)^{a}$ | $(0.07)^{a}$ | $(0.18)^{a}$ | $(0.73)^{a}$ | $(0.56)^{b}$ |
| Glycerol | 7.08 | 7.36 | 6.92 | 8.05 | 6.07 | 7.60 | 0.94 |
| (g/L) | $(0.11)^{cd}$ | $(0.08)^{cd}$ | $(0.13)^{c}$ | $(0.42)^{e}$ | $(0.50)^{b}$ | $(0.18)^{de}$ | $(0.08)^{a}$ |

The Linosa yeast strains performance was in general comparable to that of the commercial L404 strain. This shows that new yeast strains of potential interest for enology can be found in a situation of geographic isolation.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