Molecular and Enological Characterization of Autochthonous *Saccharomyces cerevisiae* Strains Isolated from Grape-musts and Wines Cannonau

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Abstract-- **Fermentation by autochthonous yeasts may produce wines with enological properties that are unique of a specific area or variety of grape must. In order to identify yeast starter strains for the production of the Sardinian wine Cannonau DOC, 66 *Saccharomyces cerevisiae* strains, isolated from musts and wines Cannonau of six vitivinicole areas in Sardinia, were subjected to enological characterization and molecular identification. The RFLP-PCR fingerprinting of the ITS region of rRNA (ITS1-5,8S-ITS2) as well as ethanol, foam, and H_2S production were analysed.**

I. Introduction

Grape juice fermentation is a complex microbiological process in which yeasts play a central role [1]. Nowadays wine fermentation is carried out by selected yeast starter strains that possess very good fermentative and oenological capabilities. Even though commercial strains have contributed to the standardization of the fermentative process, their use can lead to the loss of wine specific characters. Indeed wine aroma and bouquet is related to a large extent to the yeast strains that lead the fermentative process [2]-[3]. Traditionally wine fermentation was carried out by indigenous natural yeast flora. These yeasts have been unintentionally selected during centuries of use in specific contest (region - grape variety - oenological practices) in order to enhance the quality of specific wines. In this way is possible to explain the huge diversity in wine typology encountered in the "old" winemaking region such as Italy, France or Spain. On the contrary commercial yeast starter strains have been selected intentionally in only few contest and they are widely used even in wine of completely different typology [4].

In this work we have characterized autochthonous yeast strains that can be used as starters for the production of Cannonau DOC, the most produced red wine in Sardinia.

These yeasts have been previously isolated in different years, ranging from 1967 to 2004, in different wine producing areas not yet contaminated by commercial strains.

The aim of this work was to identify natural yeasts able to enhance the characters of specificity of the Cannonau wine. Besides, exploring the biodiversity of indigenous fermentative strains can be an important contribution towards the understanding and selection of strains with specific phenotypes.

II. Materials and Methods

Yeast strains

The 66 natural yeast strains used in this study are listed in ***Table 1***. These yeasts, belonging to the Di.S.A.A.B.A. collection of strains, were isolated in six Cannonau producing areas in Sardinia and identified by using the official taxonomic key [5]-[6] The yeast strains CBS1171 *(Sacch. cerevisiae)*, CBS380 (*Sacch. bayanus*) and CBS395 (*Sacch. uvarum*) from Centraalburau voor Schimmelcultures were used as type reference strains.

TABLE I
STRAINS USED IN THIS STUDY

Isolation's area	Isolation's medium	Isolation's year	Number of strains
Oliena	must	1967	23
		1968	3
		1988	8
Oliena	wine	1967	10
Tortolì	must	1990	1
Jerzu	wine	2004	6
Alghero	must	1988	7
Sorso	must	1967	2
Cagliari	must	1988	6

Molecular identification

Yeast molecular identification was carried out by RFLP-PCR analysis of internal transcribed spacers ITS1-5,8S-ITS2. DNA isolation was carried out from pure cultures of each strain by using the protocol "Yeast DNA miniprep (5ml)" [7] PCR amplification of the 5.8S-ITS region was performed with primers ITS1 **(5'TCCGTAGGTGAACCTGCGG3')** and ITS4 **(5'TCCTCCGCTTATTGATATGC3')** as described by White *et al.* [8] The PCR conditions were described by Esteve-Zarzoso *et al.* [3]. Aliquots of PCR products were digested with the restriction endonucleases *CfoI, HaeIII* and *HinfI* (New England BioLabs) according to the manufacturer's instructions in total volume of 20 µl [1]-[3]. Restriction fragments were analysed by electrophoresis in 2% (w/v) agarose gels. DNA molecular-weight-marker (123 bp DNA Ladder, Invitrogen) was used as standard.

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Technological and genetic characterization

For the subsequent analyses, all the yeasts strains were precoltured on YEPD medium (0.5% yeast extract, 1% bacto-peptone, 2% glucose) overnight at 25°C in incubator-shaker.

Growth at different temperatures. 5×10^6 cells (approx. 50 µl of the overnight colture) were spotted on YEPD-plates and incubated at 4°, 30° and 35°C. Growth was evaluate after 24h of incubation at 30 and 35 °C and after 4 days for growth at 4°C.

Hydrogen sulphide production. 5×10^6 cells (approx. 50 µl of the overnight colture) were spotted on Biggy agar plates (Difco). Quantification of H_2S was achieved by colony colour formation, scoring the browning degree (1-6) associated with yeast growth according to the following scale: 1 = white, 2 = cream, 3 = light brown, 4 = brown, 5 = dark-brown, 6 = black [9].

Sporulation. Sporulation was induced on K-acetate (1% potassium acetate, 2% bacto-agar) and Na-acetate (1% sodium acetate, 0.5% sodium chloride, 2% bacto-agar) plates, the frequency and efficiency of sporulation was determined as described by Mortimer *et al*. [10].

Foam production. Yeast were inoculated in test tubes (16mm x 160mm) with 8 ml steril must (cultivar Cannonau) and incubated at 25°C. Foam height was measured every day throughtout the fermentation. Yests were classified into five categories based on foam production into the tube (A: foam ring; A+:foam ring ≤ 0.5cm; C-: foam on all surface of the tube < 0.5 cm; C: foam on all surface of the tube 0,5≤ C ≤ 1 cm; C+: foam on all surface of the tube > 1cm).

Growth kinetics. Yeast cell were cultured in Cannonau grape juice and 2ml of the culture (approx. 10^6 cell ml^{-1}, grown at 25°C) were suspended into 100 ml Erlenmeyer flasks containing 50 ml of grape juice (cultivar Cannonau, pH 3,4, 19,3% w/v of reducing sugar) adjusted to a sugar concentration of 30% (w/v) by addition of glucose, and steam sterilized at 100°C for 10 min. The flasks were fitted with Müller glass valves, containing sulphuric acid, and thus allowing only CO_2 to escape from the system. Growth kinetics were determined by weight loss measurement during several days until the end of the fermentation [11]. The yeast strains tested were evaluated by determining the fermentation power (maximum amount of ethanol produced in presence of excess sugar, expressed as %v/v), fermentation rate (amount of carbon dioxide produced after 3 days of fermentation, as grams of CO_2 per day).

III. RESULTS AND DISCUSSION

Identification of yeasts

The ITS1 and ITS4 primers amplify a DNA region which includes two non-coding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. All the tested strains showed a single band of 840-880 bp (**Figure 1**), characteristic of strains belonging to the *Saccharomyces* genus [8]-[1]-[13]. Profiles obtained by the digestion of PCR products with *HaeIII* resulted in four restriction bands with sizes ranging from 325 to 125 bp for the strains identified as *Sacch. cerevisiae* and 3 restriction bands for *Sacch. bayanus* and *Sacch. uvarum* (references strains) (**Figure 2b**). Two restriction bands of 365 + 155 bp were obtained when amplicons were digested with *HinfI* and two restriction bands of 385 + 365 bp were obtained when amplicons were digested with *CfoI* as described by various authors [8]-[1]-[12]-[13] (**Figure 2b, c**).

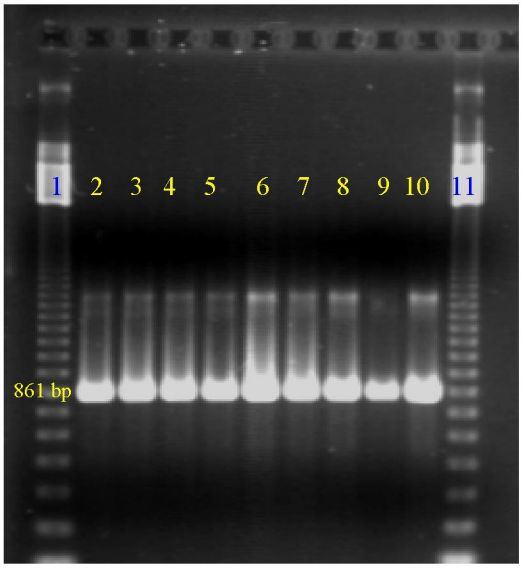
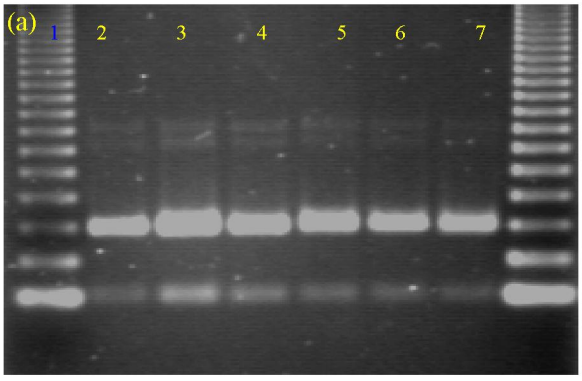


Fig. 1. Size of the PCR-amplified rDNA region of the wine strains: lanes 1 and 11 corresponds to molecular markers (123 bp DNA Ladder, Invitrogen); lanes from 2 to 10 corresponds to some strains in study.

The fingerprinting obtained with the use of the restriction enzyme *HaeIII* allowed us to identify all the natural strains tested as belonging to *Saccharomyces cerevisiae* specie. On the contrary neither *HinfI* or *CfoI* display a discriminative power at specie level as the fingerprinting obtained did not discriminate among the reference strains.



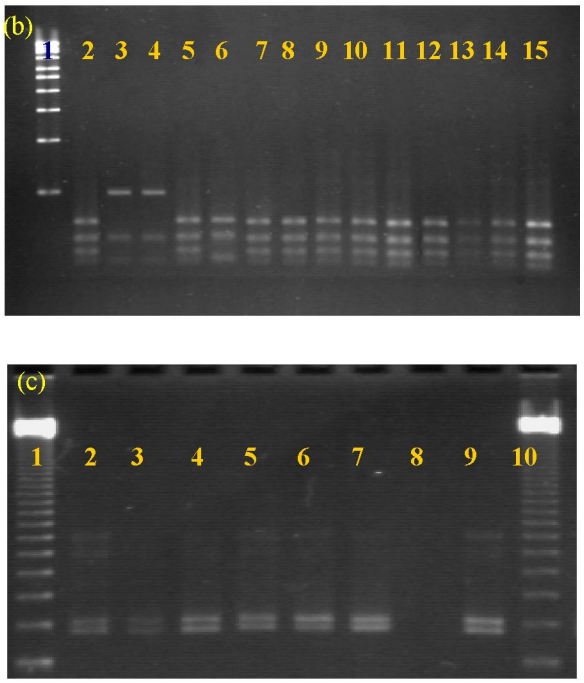


Fig. 2. Restriction analysis with the endonucleases *HinfI* (a), *HaeIII* (b) and *CfoI* (c). (a) lanes 1 and 8 corresponds to molecular size standard (123 bp DNA Ladder, Invitrogen) lanes 2, 3 and 4 correspond to the reference strains CBS1171, CBS 380 and CBS 395, lanes from 5 to 7 corresponds to some strains in study; (b) lanes 1 corresponds to molecular size standard (1Kb DNA Ladder, Sigma) lanes 2, 3 and 4 correspond to the reference strains CBS1171, CBS 380 and CBS 395, lanes from 5 to 15 corresponds to some strains in study; (c) lanes 1 and 11 corresponds to molecular size standard (123 bp DNA Ladder, Invitrogen) lanes 2, 3 and 4 correspond to the reference strains CBS1171, CBS 380 and CBS 395, lanes from 5 to 1 corresponds to some strains in study.

Thechnological and genetic characterization

In the followings **Figure 3, 4 and 5** are showed the results obtained from the growth at various temperatures, sporulation frequencies and efficiencies as well as H_2S production. 18% of the strains were able to grow at 4°C. Most of the strains revealed low production of H_2S and showed a high frequency and efficiency of sporulation.

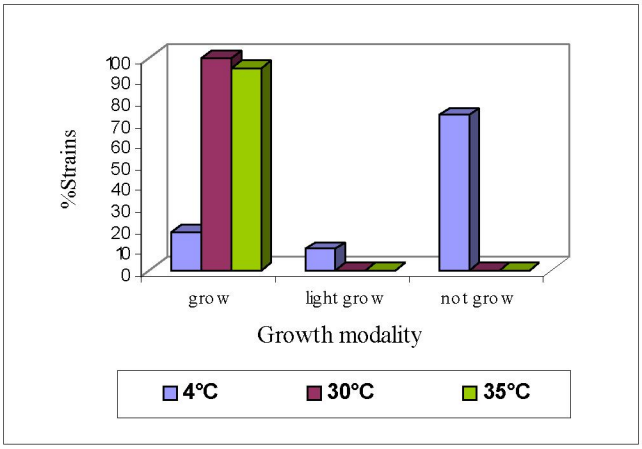


Fig. 3. Growth at different temperature: % of strains that at various temperature grew good, low or did not grow.

Hydrogen sulphide production. Volatile sulfur product such as hydrogen sulfide are found in wine as in other fermented beverages [14]. Because of their very low perception level and nauseous character, these compounds are the cause of some organoleptic defects [15]. Yeasts are directly involved in hydrogen sulphide liberation during must fermentation due to their sulfite reductase activity [16]. It is highly desiderable, therefore, to utilize yeast starter strains that produce and release very low amount of H_2S. Most of the strains tested (>50%) showed a very low production (cream and light brown colony colours) of H_2S (**Figure 4**).

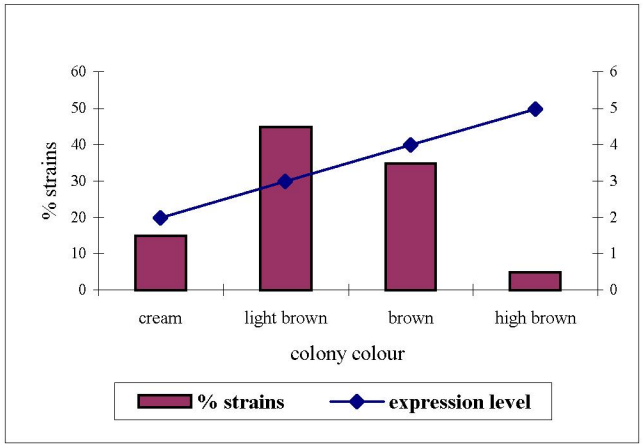


Fig. 4. H_2S production on Biggy-agar: % of strains that revealed different colony colors by the production of hydrogen sulphide

Sporulation. On Na-acetate medium the percentage (66%) of strains that sporulate is greater than on K-acetate (50%) (**Figure 5**). The percentage of frequency and efficiency of sporulation are variable on the two media: on Na-acetate medium the frequency is lower than on K-acetate, on the contrary the efficiency of sporulation is higher on Na-acetate than on K-acetate. In order to design a program of GI (Genetic Improvement), tetrad analysis is important to define strains stability of oenological characters. With regard to this, is important to analyze yeast strains with high values of sporulation frequency and efficiency and high spore viability [17]- [18].

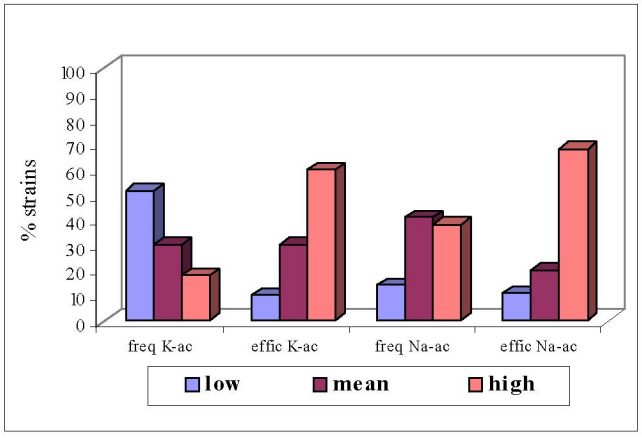


Fig. 5. Sporulation frequency and efficiency : % of strains that revelead high, mean and low frequency and efficiency in the two minimum media.

Foam production. Most of the strains showed an elevated production of foam **(Figura 6).**

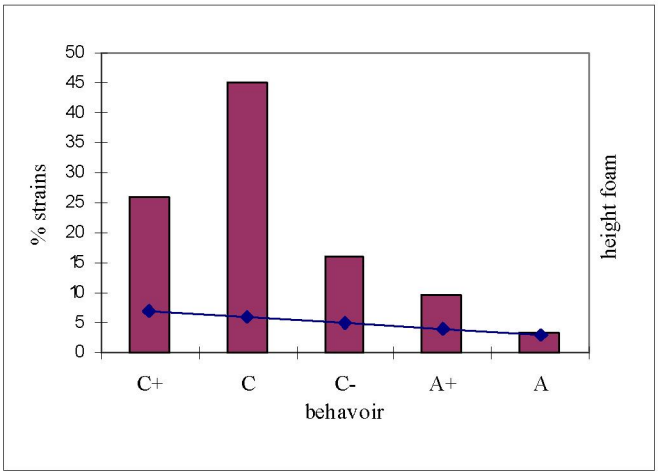


Fig. 6. Foam production: % of strains that revelead various behavior for this trait.

Growth kinetics. 75% of strains produced levels of ethanol ranging from 15 to 17 %v/v. 50% of strains showed a fermentation rate ranging between 1,15-1,35 g CO_2/day in the first three days of fermentation (**Figure** 7). This data showed that the natural yeasts tested possess a very high fermentative power and thus they are able to quickly grow and replace the undesired natural flora.

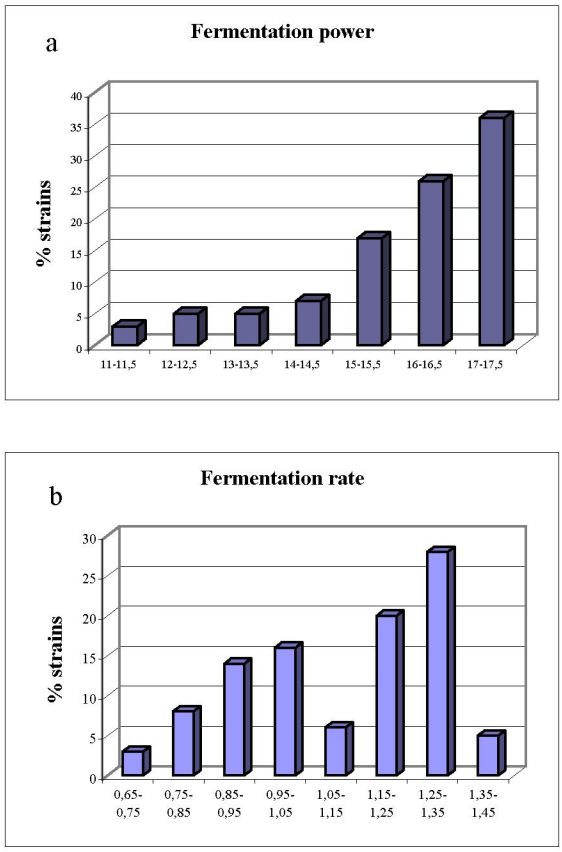


Fig. 7. Growth kinetics: (a) % of strains that revealed fermentation power ranging from 11%v/v and 17,5%v/v of ethanol production; (b) % of strains that revealed different fermentation rate expressed by g CO_2 /day.

IV. Conclusions

Data obtained in the present work allowed us to identify natural strains suitable for the production of Cannonau DOC. In particular some strains present a high fermentative power, reduced H_2S production and relatively low foam production. The use of these strains as starters for the production of the Cannonau DOC could lead to improvement in wine organoleptic characteristics such as colour, aroma, etc. In order to verify this hypothesis the best strains will be tested in a microvinification assay in a Cannonau DOC must. Besides, further analyses will be performed to better characterize the strains at molecular level by using minisatellites markers. These markers could allow to trace the yeast and thus to monitor the fermentative process. Finally natural yeast strains will be isolated in 2006 in order to homogenize the sample number in the different areas of Cannonau DOC production.

V. References

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