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A survey of *Saccharomyces* populations associated with wine fermentations from the Apulia region (South Italy)

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Abstract - The aim of this paper was to investigate the genetic and phenotypic characteristics of yeasts isolated from samples of grape musts collected from four different areas of Apulia region. The 68 yeast isolates were identified as *Saccharomyces cerevisiae* by PCR-RFLP of 5.8S-ITS region of the rRNA gene. Individual isolates were differentiated by RAPD-PCR and AFLP. The following oenological traits were studied: fermentation power, resistance to cycloheximide, alcohol and SO_2, formation of SO_2 and H_2S, β-glucosidase activity, and production of biogenic amines and secondary compounds. Many phenotypes were common to several yeasts isolated from the four different areas, such as high SO_2 resistance and fermentation power. In addition, some *S. cerevisiae* isolates showed a β-glucosidase activity and others had a high resistance to cycloheximide. All the strains formed biogenic amines. Solid Phase Microextraction was used to determine secondary compounds produced in wine by the single yeast cultures.

Key words: spontaneous wine fermentation; *Saccharomyces cerevisiae*; biodiversity; volatile compounds; biogenic amines.

INTRODUCTION

The composition of the yeast biota can vary according to the climatic conditions, the grape variety and the vinification technology. Because of this variability, several aspects of microbial ecology present in wine fermentations warrant investigation.

It is well known that the unique flavour of each wine is the result of multivariate interactions between the geography, climate, cultivar, vineyard management and oenology. Thus in recent years, much work has been carried out on the ecology of wine yeasts, in order to ascertain the existence of strains typical of one ecosystem and also to carry out selection programmes of the yeasts representative of each wine-producing zone (Frezier and Dubordieu, 1992; Vezinhet *et al*., 1992; Gutierrez *et al*., 1999; Romano *et al*., 2003; Cappello *et al*., 2004; Martinez *et al*., 2004).

Some authors have stated that the aromatic wine properties can be deeply affected by the presence of higher alcohols and other volatile substances produced by yeasts during must fermentation (Antonelli *et al*., 1999; Domizio *et al*., 2007). The quantitative production of secondary aromatic compounds of fermentation is of great interest to enhance the varietal character of the wine and optimise winemaking, as well as the enzymatic activity of yeasts, such as esterases (Rosi and Bertuccioli, 1989), β-glucosidase (Rosi *et al*., 1994), proteinase (Ubeda Iranzo *et al*., 1998) and pectic enzymes (Gainvors *et al*., 1994). However, these enzymatic activities are not distinctive of a particular genus or species, but depend on the yeast strain analysed. Some of these strains may be responsible for giving the wine the unique characteristics from the region of its production (Pretorius, 2000). A large variability in the molecular polymorphisms of natural *Saccharomyces cerevisiae* wine yeasts has also been found (Vezinhet *et al*., 1992; Nadal *et al*., 1996; Querol *et al*., 2003; Ayoub *et al.,* 2006). The natural yeast biota seems to be made up of relatively small, genetically isolated yeast subpopulations that can be distinguished by both their genetic markers and their phenotypic characteristics (Nadal *et al*., 1996). These differences could reflect an adaptation to specific pedo-climatic conditions of vineyards or cellar microenvironments.

This study was undertaken to evaluate the genetic and biotechnological characteristics of the indigenous *Saccharomyces sensu stricto* populations from musts of different areas of the Apulia region. The representative yeast biotypes, that can be individualised, would be useful as inoculants in vinifications carried out in that specific oenological area.

MATERIALS AND METHODS

Sampling. Sampling was carried out in wineries located in four different areas (AS, BS, FS and GS) of the Apulia region, South Italy (Fig. 1). Sampling was done in both white and red musts immediately after grape crushing

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without addition of SO_2 and active dry yeasts. The musts (2 l for each sample) were spontaneously fermented in the laboratory at 25 °C and at the end of fermentation were plated out on YEPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} glucose) for single colony isolation. A total of 68 colonies randomly selected from plates were isolated and maintained on the same medium at 4 °C for further investigations.

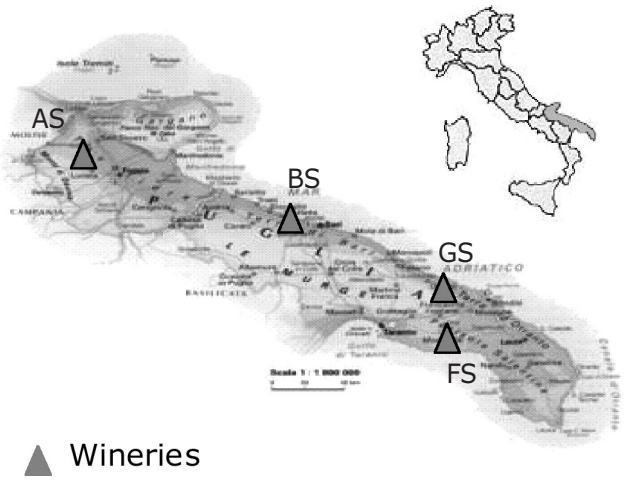

FIG. 1 - Location of wineries in four different areas (AS, BS, FS and GS) of the Apulia region, South Italy.

Molecular identification and typing of yeasts. Four type strains of the *Saccharomyces sensu stricto* group, namely *Saccharomyces bayanus* DBVPG 6171^T, *Saccharomyces cerevisiae* DBVPG 6173^T, *Saccharomyces paradoxus* DBVPG 6411^T and *Saccharomyces pastorianus* DBVPG 6047^T, obtained from the Industrial Yeasts Collection of Perugia (Italy), were used as reference strains for the molecular characterisation of the isolates.

Total genomic DNA was extracted and purified from 7-ml cultures as described by Querol *et al*. (1992). Quantification of total DNA was achieved using a VersaFluor fluorimeter and a Fluorescent DNA Quantitation Kit (Biorad, USA).

Identification of the isolates was ascertained by PCR-RFLP of 5.8S-ITS region of the rRNA gene as described by Esteve-Zarzoso *et al*. (1999). The PCR fragments were digested with 1 U of *Hae*III and *Hinf*I endonucleases (Takara Shuzo Co., Otsu, Shiga, Japan) for 2 h at 37 °C. Restriction products were analysed on a 2% agarose gel. RAPD-PCR assay was carried out using primer LA1, 5′ GCGACGGTGTACTAAC 3′, and the amplification conditions described by de Barros Lopes *et al*. (1996), except for the extension temperature of the amplification cycle (72 °C instead of 74 °C). Products of the RAPD-PCR reaction were resolved on a 1.5% agarose gel and stained with ethidium bromide.

AFLP reaction was conducted as described by de Barros Lopes *et al*. (1999), with some modifications. Briefly, DNA (0.5 mg) was digested with 10 U *Eco*RI and 10 U *Mse*I for 2 h at 37 °C and 65 °C, respectively. *Eco*RI (0.04 pmol 5′ CTCGTAGACTGCGTACC 3′ and 5′ AATTGGTACGCAGTC 3′) and *Mse*I (0.4 pmol 5′ GACGATGAGTCCTGAAG 3′ and 5′ TACTCAGGACTCAT ‘3) adaptors were ligated to the digested DNA in a total volume of 30 ml using 1 U T4 DNA ligase. After incubation at 37°C for 3 h, the digested and ligated DNA was precipitated and resuspended in 50 ml Tris 10 mol l^{-1}-EDTA 0.1 mol l^{-1} buffer (pH 8). The following oligonucleotides were used for selective PCR amplification: *Eco*RI-C(5′AGACTGCGTACCAATTCC3′) and *Mse*I-AC (5′GATGAGTCCTGAGTAAAC 3′). For each reaction (20 ml), 2 ml of the ligated DNA was amplified using 1 pmol *Eco*RI-C and 5 pmol *Mse*I-AC, 1.5 mol l^{-1} $MgCl_2$, 0.2 mol l^{-1} dNTPs and 0.1 U *Taq* polymerase (Polymed, Florence, Italy). A "touchdown" cycle was used for the PCR amplification: denaturation was at 94 °C for 1 min and extension at 72 °C for 5 min; the annealing temperature started at 64 °C and was decreased until 56 °C. This was followed by 10 more cycles at 56 °C and a final 5 min extension at 72 °C. Products of each reaction were heated to 95 °C for 3 min and cooled on ice. The selective PCR products were resolved on a denaturing 6% (w/v) polyacrylamide gel (SequaGel 6, National Diagnostics, USA) at a voltage of 250 V for 2 h, in a 1x TBE buffer (89 mol l^{-1} Tris-borate, 2 mol l^{-1} EDTA pH 8) maintained at 50 °C. Staining of the gel was performed with ethidium bromide.

RAPD-PCR and AFPL patterns were digitally saved and analysed with the GelCompar 4.0 Software (Applied Math, Kortrijk, Belgium) using the Dice coefficient and UPGMA as calculation and clustering options.

Physiological characterisation of yeasts. The isolates were characterised following the usual criteria for spore formation and physiological tests according to Barnett *et al*. (2000). Their ability to ferment glucose, maltose, sucrose, galactose and melibiose as carbon sources was determined in Durham tubes, containing YEPD with 2% of the appropriate sugars, after 7 days at 25 °C. The capacity to grow at 37 °C was determined after 7 days in YEPD medium. The β-glucosidase activity was tested by the ability to split arbutin on arbutin agar after 3 and 7 days. The medium developed a dark brown colour when the strains hydrolysed arbutin. The production of H_2S was determined by the yeast colour formation on BIGGY agar plates (Difco, Detroit, MI, USA), scoring the browning degree (1-4) associated with yeast growth. The production of SO_2 was evaluated in a basal synthetic medium as reported by Romano *et al*. (1992). The ability of cultures to grow in the presence of different concentrations of sulphur dioxide (50, 100 and 150 ppm), ethanol (5, 10, 15 and 18%), and cycloheximide (0.01 and 0.05 w/v) was also determined as described by Paraggio *et al*. (1998).

Microvinification trials. Fermentation of 100 ml aliquots of white grape must from Trebbiano cultivar with 18% (w/v) fermentable sugars, 0.74% (w/v) titratable acidity, pH 3.2 was carried out in 130 ml Erlenmeyer-flasks. The must samples, after treatment at 100 °C for 30 min, were inoculated with 5 ml of 48 h precultures grown in the same must. Alcoholic fermentations were conducted at 25 °C for 15 days by determining the weight loss caused by CO_2 evolution. When the CO_2 release ceased, the fermentation was considered completed and the samples were refrigerated for 2 days at 4 °C, racked and stored at −20 °C until analysis. Two fermentations were carried out with each strain. In all determinations must was used as a control.

Biogenic amine production. Isolates of *S. cerevisiae* were screened for the capacity to produce biogenic amines according to the method proposed by Bover-Cid and Holzapfel (1999). All strains were streaked in duplicate on the decarboxylase medium plates with and without amino acids (control) and were incubated for 4 days at 25 °C, under aerobic and anaerobic conditions (AnaeroGen, Oxoid). Positive reactions were recorded when a purple colour occurred around the colonies.

For determination of the biogenic amines produced by single isolates of *S. cerevisiae* in wine, 0.5 ml of each sample were derivatized using the method of Eerola *et al*. (1993) and submitted to HPLC analysis according to Galgano *et al.* (2001). The chromatographic system consisted of a Spectra System P4000 pump, a Spectra System AS3000 Autosamples, a Spectra System UV1000 UV/VIS detector (ThermoFinningan Italia spa, Rodano, Italy) and a personal computer running the chromatographic software ChromQuest for Windows (ThermoQuest Italia spa, Rodano, Italy). The sample (10 ml) was injected onto a C18 Spherisorb S3ODS2 (Waters spa, Vimodrone, Italy), equipped with a Spherisorb S5ODS2 guard column (Waters).The analyses were performed in triplicate and data are expressed as mean values ± standard deviation.

Solid phase microextraction-gas chromatography (SPME-GC) analysis of volatile compounds. Volatile compounds produced by each strain were determined with solid phase microextraction (SPME) coupled with gas chromatography according to De la Calle-Garcia *et al*. (1996) and Vas *et al*. (1998). The fiber used for SPME was coated with a polyacrylate layer of 8.5 mm thickness (Supelco, USA). For quantitative determination, a CP 380 capillary gas chromatograph equipped with a 8200 autosampler SPME III (Varian, Italy) was utilised. The fused silica capillary column was a CP-Wax 52 CB (50 m x 0.32 mm) by Crompack (The Netherlands), coated with polyethyleneglycol (film thickness 1.2 mm), as stationary phase. The injector and FID temperature was 250 °C. The temperature program was the following: initial temperature (50 °C) held for 2 min; first ramp, 1 °C min to 65 °C (0 min hold); second ramp, 10 °C min to 150 °C (10 min hold); third ramp 10 °C min to 200 °C (1 min hold). The carrier gas (N_2) flow rate was 2.5 ml min.

The aroma compounds (ethyl acetate, isobutanol, n-propyalcohol, isoamyl alcohol, and 2-phenylethyl alcohol), were identified by comparing the retention time of standards and their identification was confirmed by using GC-MS. The quantitative analysis of wine aroma compounds was carried out on the basis of the relative peak area calculated from head space SPME (HS/SPME) gas chromatograms after addition of know amounts of analyte standards, as well as the internal standard according to De la Calle-Garcia *et al*. (1998).

Statistical analysis. One-way analysis of variance and Least Significant Difference were used to analyse mean differences in mean values, if any, at 95 and 99% accuracy level. Principal Component Analysis (PCA) was used in order to group homogeneous samples.

RESULTS

Isolation and phenotypic characterisation of *Saccharomyces* wine yeasts

A total of 68 independent clones from white and red wine samples from different cellars in four areas (AS, BS, FS and GS) of Apulia region were isolated at the end of fermentation on YEPD plates: 18 from area AS, 36 from BS, 6 from FS and 8 from GS. According to standard assays based on fermentation and assimilation of different carbon and nitrogen sources (Barnett *et al*., 2000) the isolates were presumptively identified as *Saccharomyces cerevisiae*.

In synthetic medium, most of the strains produced SO_2 ranging from 6.2 to 12.8 mg l^{-1} but only 5 strains (AS5, AS11, AS14, AS15 and BS24) from 20.0 to 25.5 mg l^{-1}. Moreover all strains of AS and FS were able to produces hydrogen sulphide but only 24 strains of BS and 1 strain of GS showed this character. On the other hand, only the strains belonging to AS group were sensitive to 15% ethanol. In addition, 73% of the strains developed in presence of 150 ppm SO_2.

The β-glucosidase activity measured as hydrolysis of arbutin was detected in 9 strains AS and 3 strains GS. Three strains AS were resistant to 0.05% cycloheximide.

Yeast identification and differentiation by molecular genetic methods

Species identification of the 68 indigenous yeast isolates was performed by PCR-RFLP of 5.8S-ITS region of the rRNA gene. The restriction profiles were compared with the type strains, as described in materials and methods. All yeasts showed the same and typical *S. cerevisiae* restriction patterns using the endonucleases *Hae*III and *Hinf*I separately on the PCR-product of 880 bp (Fig. 2). In fact, for *Hae*III digestion, isolates yielded fragments of 320, 230, 180 and 150 bp, while for *Hinf*I digestion, fragments of 365 and 155 bp were observed on gel. On the basis of these results, all isolates were assigned to the species *S. cerevisiae*.

To collect information at the intraspecific level, the *S. cerevisiae* isolates were subjected to RAPD-PCR and AFLP. The RAPD-PCR profiles and the cluster analysis of the 68 yeasts are reported in Fig. 3. An ample genetic polymorphism between isolates was observed. At a similarity coefficient of 83% four main groups of isolates were discerned. Clusters II and III contained almost all of the isolates (29 isolates in cluster II and 23 in cluster III). Within these clusters several subgroups were distinguishable, as well. Only strains isolated from AS area were grouped together (89% similarity).Two more clusters, I and IV, were constituted by 8 and 5 isolates, respectively. *Saccharomyces cerevisiae* BS21 and BS24 remained ungrouped.

AFLP was performed on 25 representative *S. cerevisiae* isolates chosen on the basis of these molecular clustering results. The AFLP profiles and the cluster analysis of the indigenous isolates are reported in Fig. 4. Also in this case a wide genotypic diversity was underlined between the yeasts. The two fingerprinting techniques applied displayed different discriminative power and furnished complementary results useful for the differentiation of the isolates.

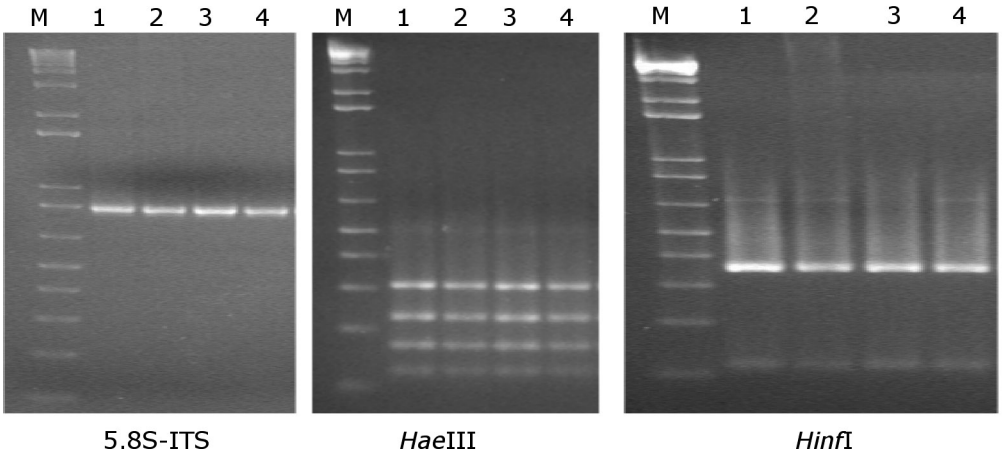

FIG. 2 - PCR-RFLP of 5.8S-ITS region of the rRNA gene for species identification of yeast isolates. M, 1 Kb plus DNA ladder marker. Lanes 1-4, indigenous isolates.

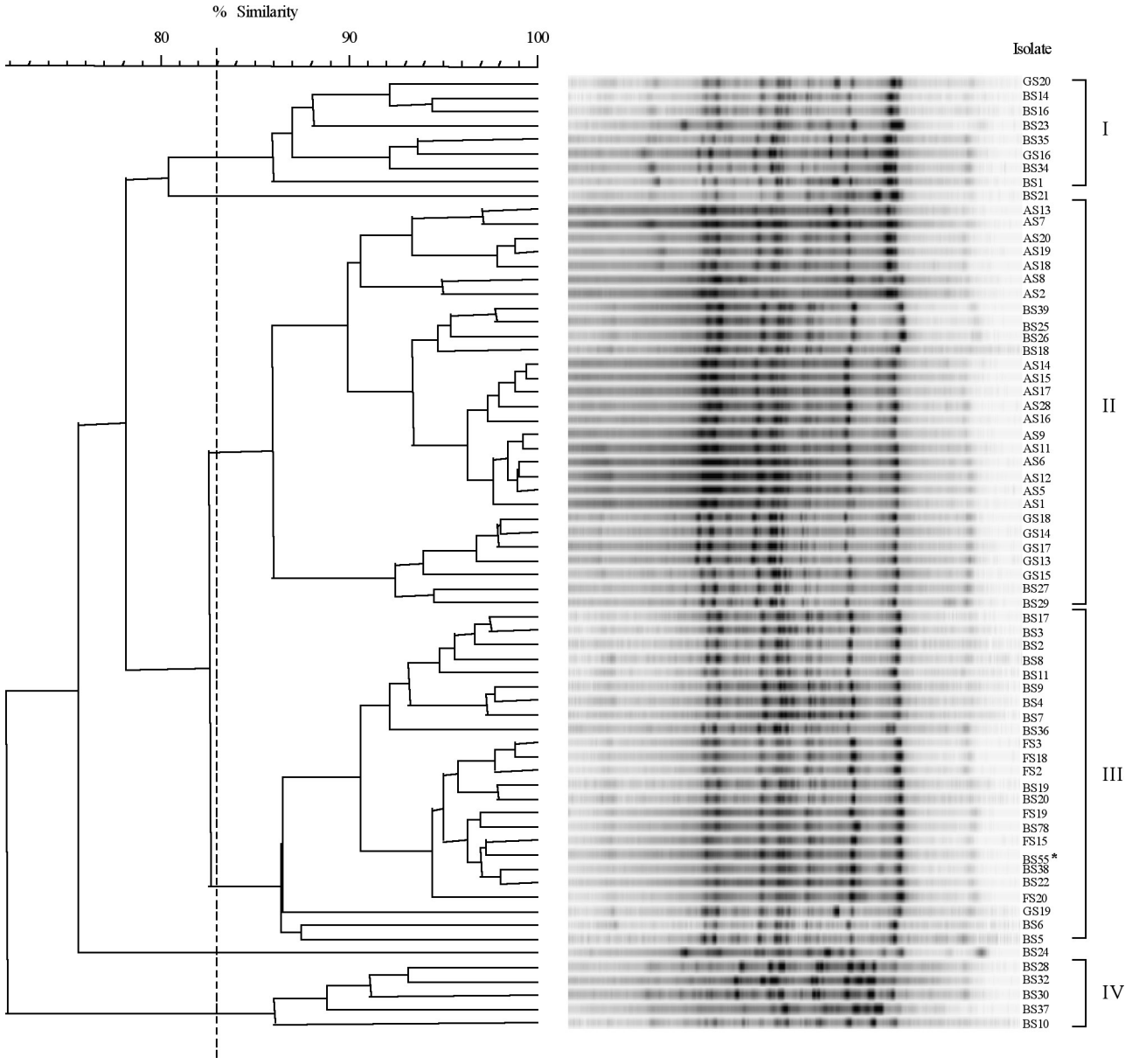

FIG. 3 - RAPD-PCR cluster analysis of profiles obtained from *Saccharomyces cerevisiae* isolates. The first letter on the strain code represents the sample, and the number represents the progressive number of isolation.

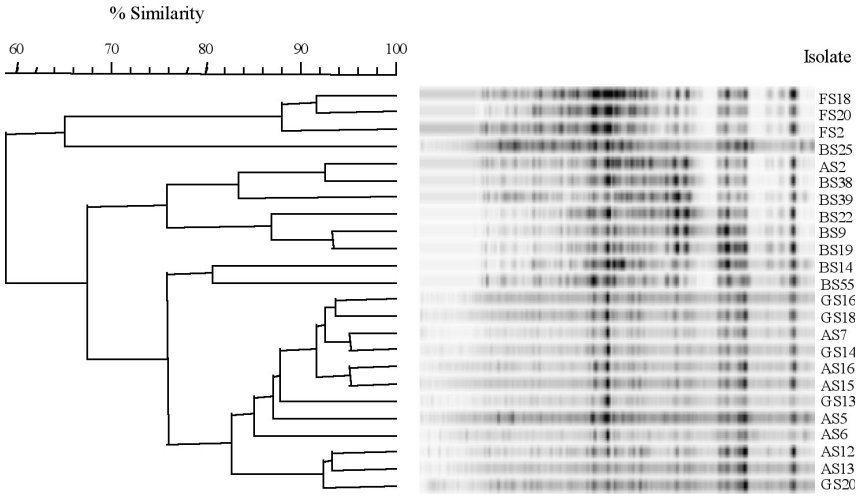

FIG. 4 - AFLP cluster analysis of profiles obtained from representative *Saccharomyces cerevisiae* isolates.

Metabolic profiles of *Saccharomyces cerevisiae* isolates in wine

Trebbiano must fermentations were carried out to determine the yeast fermentation power behaviour and the secondary volatile compounds produced by the isolates from the four different areas of Apulia. During must fermentation all the yeasts produced similar amounts of ethyl acetate, isobutanol and isoamyl alcohol (Fig. 5). Moreover the isolates GS produced very high quantities of n-propanol, followed by the isolates FS while the isolates AS produced the highest quantities of 2-phenylethyl alcohol. The results obtained by two replicates of the aromatic secondary compounds were analysed by PCA (Fig. 6). The first two principal components accounted 41.53% and 27.44% of the variance. The volatile compounds ethyl acetate, isobutanol, n-propyl alcohol and isoamyl alcohol were correlated with Factor 1, while 2-phenyl ethanol with Factor 2. The wines GS formed a separated group (right half of the plane) and wines AS, BS, and FS appeared a close group. This separation in two well-defined groups of wines would be related with the origin of the strains.

The screening for biogenic amine production evidenced that all the *S. cerevisiae* isolates were amino acid decarboxylase positive. Thus, three strains for each group were tested for biogenic amine production in must. As show in Table 1, all the *Saccharomyces* produced ethylamine and putrescine, whereas phenylethylamine, cadaverine, histamine, spermine and spermidine production were strain dependent. Similar results have been reported by Caruso *et al*. (2002).

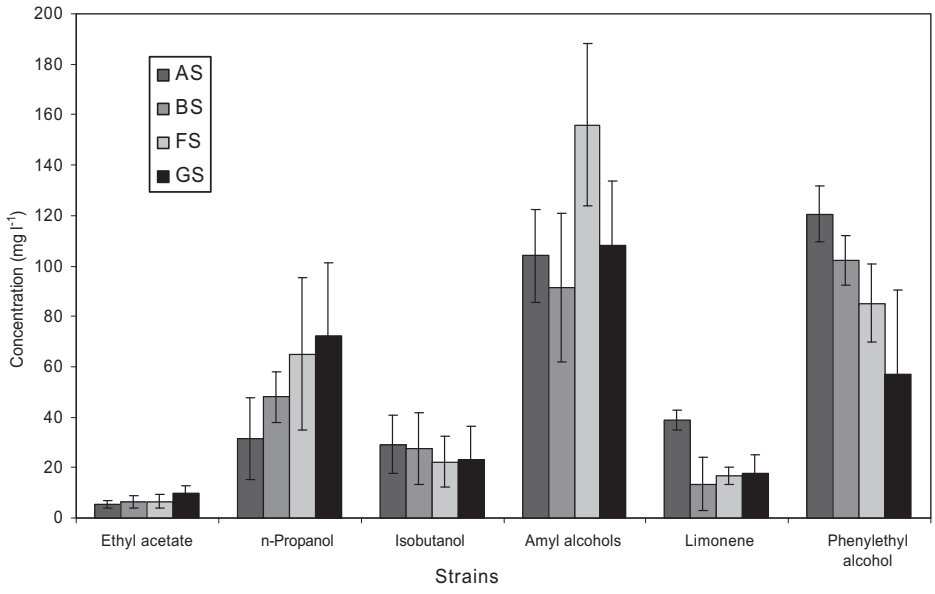

FIG. 5 - Mean and standard deviation of volatile compounds (mg l^{-1}) produced by the *Saccharomyces cerevisiae* isolates from the four areas of the Apulia region.

TABLE 1 - Biogenic amine (mg l^{-1}) produced in must by the *Saccharomyces cerevisiae* yeasts isolated from musts S, BS, FS and GS at the final stage of fermentation

Isolate	Biogenic amines* (mg l^{-1})							
	ETH	TRP	PHE	PUT	CAD	HIS	SPD	SPM
AS area								
AS5	6.95 ± 0.41	n.d.**	n.d.	1.64 ± 1.00	3.98 ± 0.70	0.15 ± 0.12	0.35 ± 0.09	n.d.
AS7	2.50 ± 2.87	n.d.	1.04 ± 1.47	5.18 ± 0.20	3.99 ± 2.54	0.06 ± 0.09	n.d.	n.d.
AS9	0.53 ± 0.74	n.d.	0.97 ± 0.07	5.82 ± 0.14	4.48 ± 0.16	n.d.	n.d.	n.d.
BS area								
BS4	2.08 ± 0.35	n.d.	1.06 ± 0.01	1.72 ± 0.02	0.32 ± 0.45	0.12 ± 0.17	n.d.	n.d.
BS17	2.58 ± 0.07	n.d.	0.17 ± 0.03	4.36 ± 0.05	6.91 ± 0.30	1.06 ± 0.04	1.47 ± 0.05	0.35 ± 0.05
BS19	3.62 ± 2.76	n.d.	n.d.	2.14 ± 0.29	n.d.	n.d.	0.65 ± 0.91	n.d.
BS25	1.17 ± 0.15	n.d.	0.43 ± 0.27	2.46 ± 0.44	n.d.	n.d.	0.49 ± 0.51	0.05 ± 0.07
BS32	1.75 ± 0.49	n.d.	0.80 ± 0.14	2.41 ± 0.71	n.d.	n.d.	0.16 ± 0.22	n.d.
BS39	0.89 ± 0.15	1.02 ± 0.04	0.68 ± 0.04	2.90 ± 0.04	3.22 ± 0.02	1.88 ± 0.01	0.60 ± 0.03	0.96 ± 0.02
FS area								
FS1	18.81 ± 24.68	n.d.	0.10 ± 0.14	3.06 ± 0.97	11.98 ± 3.64	n.d.	0.53 ± 0.75	n.d.
FS6	1.78 ± 0.13	n.d.	n.d.	2.86 ± 0.27	9.44 ± 5.41	0.31 ± 0.44	0.35 ± 0.49	0.71 ± 1.00
FS20	2.20 ± 0.52	0.44 ± 0.62	n.d.	2.93 ± 0.16	8.76 ± 0.26	n.d.	0.35 ± 0.50	n.d.
GS area								
GS13	4.75 ± 5.72	n.d.	n.d.	2.49 ± 0.30	4.80 ± 1.42	0.07 ± 0.10	0.24 ± 0.35	0.09 ± 0.12
GS11	8.41 ± 2.95	n.d.	0.25 ± 0.08	3.20 ± 0.42	3.78 ± 0.54	n.d.	0.53 ± 0.23	n.d.
GS17	10.50 ± 10.86	n.d.	n.d.	1.55 ± 0.47	n.d.	n.d.	n.d.	n.d.

* Ethylamine (ETH), tryptamyne (TRP), 2-phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), spermidine (SPD), spermine (SPM); tyramine was not detectable for all isolates; ** n.d., not detectable.

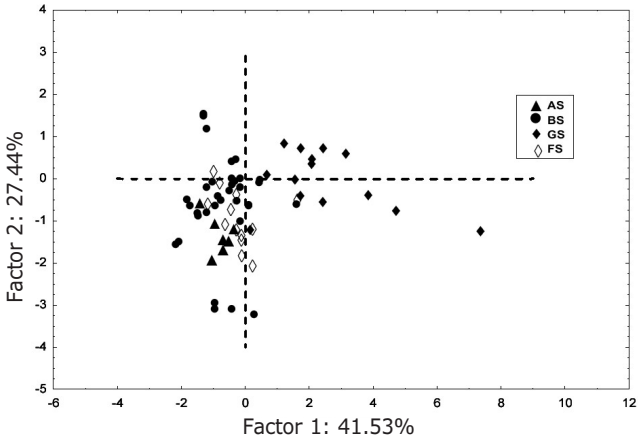

FIG. 6 - Principal Component Analysis of the volatile compounds of wines fermented by the *Saccharomyces cerevisiae* isolates from the four areas of the Apulia region.

DISCUSSION

A number of researches on the ecology of wine yeasts have allowed to establish the existence of typical strains belonging to one ecosystem and to select yeasts representative of a specific wine-producing area. In this study, *S. cerevisiae* strains isolated from different wine-producing areas of the Apulia region showed interesting biotechnological characteristics, such as cycloheximide resistance, low H_2S production, β-glucosidase activity, and high resistance to ethanol. In fact, usually, *S. cerevisiae* strains isolated from nature are very sensitive to the antibiotic cycloheximide, being inhibited by 1-1.5 μg ml^{-1} (Del Pozo *et al*., 1991) and the frequency of spontaneous mutation to cycloheximide resistance is extremely low (less than 10^{-8}). This mutation did not affect the fermentation kinetics, the quality of the wines, or the viability of active dry yeast made with the mutants (Perez *et al*., 2000) for this reason *S. cerevisiae* has been proposed also to monitor must fermentation. With respect to β-glucosidase activity, many strains of the area AS were able to hydrolyse arbutin. This enzymatic activity is interesting from an oenological point of view, since it plays an important role in determining the flavour of grapes and wines by the release of monoterpenes (Dubourdieu *et al*., 1988). Similarly, the low or absent production of H_2S by the *S. cerevisiae* isolated from areas FS and GS can be considered a positive characteristic for wine quality. According to De Simone *et al*. (1994), this characteristic was associated to the higher content of n- propanol in the wines produced by the strains GS. The well-documented technological variability within the *S. cerevisiae* species (Rankine, 1968; Soufleros and Bertrand, 1979; Cabrera *et al*. 1988; Romano and Suzzi, 1993; Romano, 1998; Pretorius, 2000; Romano *et al*. 2003) was also confirmed for the *S. cerevisiae* isolates from the Apulia region, which showed a great variability in the phenotype and in the metabolic profile.

The technological and ecological characteristics of the isolates from Apulia Region might be related to a different strain capability to adapt and colonise the vineyards and cellars, thus becoming representative biotypes. The molecular approaches applied in this study allowed to recognise genetically different strains of *S. cerevisiae*, which were clustered

in subgroups related to the four different wine-producing areas of the Apulia region, thus confirming the phenotypic results. Relationships between genetic diversity of yeast populations and ecological origin have been previously demonstrated (Vezinhet *et al*., 1992; Querol *et al*., 2003).

In conclusion, the results of this study confirmed that the geographical origin of the strains can be reflected in the production of secondary compounds and consequently in the sensory quality of the wine. As a consequence of this, the individuation of phenotypic and genotypic characteristics of yeast performing must fermentation assumes a technological importance, suggesting that the individuation of strains of biotechnological interest might derive also from an investigation of the metabolic features that exist in wild *S. cerevisiae* populations.

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