Genetic Diversity of Indigenous *Saccharomyces sensu stricto* Yeasts Isolated from Southern Croatia

Andrea SKELIN [1(✉)]
Sanja SIKORA [1]
Sandi ORLIĆ [1]
Lejla DURAKOVIĆ [2]
Sulejman REDŽEPOVIĆ [1]

Summary

Alcoholic fermentation is a polimicrobial process involving a large number of genera and species of yeast and bacteria. The major yeast genera involved in this process belongs to the *Saccharomyces* spp. The aim of this study was the isolation, identification and determination of genetic diversity of indigenous *Saccharomyces cerevisiae* natural population taken from cv. Plavac mali from secluded wine growing areas of Southern Croatia. Must samples were taken during the spontaneous alcoholic fermentation followed by yeast isolation. A total of 40 isolates that were physiologically confirmed to belong to the *Saccharomyces sensu stricto* group were furthermore differentiated by molecular methods. PCR-RFLP analyses of the internal transcribed spacer (ITS1) region of the 18S ribosomal DNA identified 37 of the isolates as *S. cerevisiae* and two of the isolates as *S. bayanus/pastorianus*. All isolates were further analyzed by RAPD fingerprinting. The results of this study showed that in some cases the RAPD assay may be useful to separate species within the *Saccharomyces sensu stricto* group. The molecular analysis confirmed genetic diversity of *S. cerevisiae* indigenous population and additionally the involvement of indigenous *S. paradoxus* and *S. bayanus* was determined. The population structure of *Saccharomyces cerevisiae* has indicated that each vineyard is characterized with particular *S. cerevisiae* microflora. It is an important step towards the preservation and exploitation of yeast biodiversity in Southern Croatia.

Key words

wine yeasts, spontaneous fermentation, *Saccharomyces sensu stricto*, PCR-RFLP, RAPD fingerprinting

[1] University of Zagreb, Faculty of Agriculture, Department of Microbiology, Svetošimunska 25, 10000 Zagreb, Croatia
✉ e-mail: andreasv@agr.hr
[2] University of Zagreb, Faculty of Food Technology and Biotechnology, Department of Biochemical Engineering, Laboratory for General Microbiology and Food Microbiology, Pierottijeva 6, 10000 Zagreb, Croatia

Received: April 7, 2007 | Accepted: January 31, 2008

ACKNOWLEDGEMENTS
This work was supported by grant 0178047 from the Croatian Ministry of Science, Education and Sports.

Introduction

Grapes are primary source of yeasts in wine fermentations. They represent a natural source of strains and species biodiversity contributing to the basic chemical structure and organoleptic characteristics of the final product. Wine fermentation is either spontaneously without inoculation or by inoculation of the grape juice with selected starter cultures. Mixed populations of wild indigenous yeasts have sequential development through the process of spontaneous fermentation (Fleet, 2003). The early stages of alcoholic fermentation are dominated by the growth of non-*Saccharomyces* yeasts with relatively weak fermentative capacity. However, they are numerically superior and contribute to the fermentation since they can reach populations of about 10^6 – 10^7 cells m/L. Sensitivity to the ethanol limits their growth and they are gradually overgrown by *Saccharomyces* species. Under conditions where sugar and alcohol are present in relatively high concentrations, *S. cerevisiae* and related species become dominant, carry out and complete the process. The *S. cerevisiae* strains differ significantly in their fermentation performance and their influence upon wine aroma giving desirable sensory characteristics (Romano et al., 1998). In the last decade, the application of several molecular techniques has generated a large number of studies concerning ecology and biodiversity of indigenous *Saccharomyces cerevisiae* strains (Schuller et al., 2004). The restriction fragment length polymorphism (RFLPs) of PCR-amplified fragments from the rDNA gene cluster has proved to be fast and powerful technique for the identification of yeasts at the species level (Valente et al., 1996; Smole Možina et al., 1997; Dlauchy et al., 1999). In the present study, PCR-RFLP of the 18S rDNA + ITS1 were used for delineation of sibling species within *Saccharomyces sensu stricto* group. However, PCR-RFLP does not allow the discrimination of different strains within the same species. Therefore, individual strains were identified using RAPD.

The main aim of these investigations was isolation, identification and determination of genetic variability of the indigenous *Saccharomyces cerevisiae* population taken from *cv.* Plavac mali from secluded vine growing areas of the Pelješac peninsula as well as from the island Hvar. Plavac mali (*Vitis vinifera* L.) is an old and the most important red wine variety of Croatia which was always considered to be an indigenous Croatian variety. 'Plavac mali' is grown in the viticulture regions of Middle and South Dalmatia, where it is the recommended variety in most vine growing regions. 'Plavac mali' grapes attain superior quality in those localities that face the sea and get the most sunshine, direct and reflected, such as Dingač vineyards on Pelješac peninsula and Sv. Nedjelja vineyards on island Hvar.

Materials and methods

Yeast isolates and reference strains

During the harvest 2000 grape samples of the Plavac mali cultivar were taken from secluded vine growing positions of Southern Dalmatia (Dingač, Pelješac peninsula and Sv. Nedjelja, island Hvar). Indigenous yeasts were isolated onto YPG agar (yeast extract 10 g/L, glucose 20 g/L, bacteriological peptone 10 g/L) from grape must samples at four different stages of spontaneous alcoholic fermentation: initial (0 vol % ethanol), middle (6 vol % ethanol), end (13.4 vol % ethanol) and sediment sampling during the racking. A selective Lysine medium (Oxoid, Basingstone, UK) was used to distinguish between *Saccharomyces* and non-*Saccharomyces* yeasts. *Saccharomyces* species were unable to grow on this medium (Angelo et al., 1987). Must sampling was followed by yeast isolation. The investigation included 40 isolates from complete spontaneous fermentation (Table 1).

Table 1. Origin and designation of *Saccharomyces sensu stricto* isolates used in this study

Site	Phase I	Phase II	Phase III	Phase IV
Hvar (Sv. Nedelja)	H1 – H5	H6 – H10	H11 – H15	H16 – H20
Pelješac (Dingač)	D1 – D5	D6 – D10	D11 – D15	D16 – D20

Morphological and physiological characteristics of isolates

The methods used for the identification of isolates were according to the conventional ones based on morphological and physiological properties. For the identification of sibling species within the *Saccharomyces sensu stricto* group, the isolates were tested on the basis of taxonomic key for the genus *Saccharomyces* proposed by Vaughan-Martini and Martini (1993). All isolates were compared with corresponding type strains, *S. cerevisiae* DBVPG 6173 (CBS 1171), *S. paradoxus* DBVPG 6411 (CBS 432), *S. bayanus* DBVPG 6171 (CBS 380) and *S. pastorianus* DBVPG 6047 (CBS 1538). Those strains that were characterized as *Saccharomyces* by these methods were furthermore analyzed by molecular methods.

PCR-RFLP of 18S rDNA + ITS1

The18S rDNA+ITS1 region was amplified in a GeneAmp PCR System 2700 (Applied Biosystems, USA) using NS1 (5'–GTAGTCATATGCTTGTCTC-3') and ITS2 (5'–GCTGCGTTCTTCATCGATGC-3') primers already described by White (1990). PCR amplification was carried out according to Redžepović et al. (2002). PCR amplicons were digested with the restriction endonucleases, namely *Hae*III and *Msp*I (Roche Diagnostics, Mannheim, Germany) as

recommended by the manufacturer. Restriction fragments were separated by submerged gel electrophoresis on precast 6 % poly (NAT) gels run in SEA apparatus (Elchrom Scientific, Cham, Switzerland) for 2.5 h at 7 V/cm and 20 °C. Gels were stained with ethidium-bromide, UV- analyzed and documented on Polaroid film type 667. Molecular size markers were run in all gels, and they included a 1 kbp DNA ladder (Life Technologies), as well as 100 bp ladder (Gensura Laboratories, San Diego, CA).

RAPD fingerprinting

Four arbitrarily chosen decameric primers containing 70 % of GC were used for strain identification. Primer sequences were as follows: 5'-GCTCGTCGCT-3' (KV1), 5'-GATCGGACGG-3' (P2), 5'-GATCGGAGCG-3' (P3), 5'-GATCGCCTGG-3' (P6). All the primers used in this work were obtained from Microsynth (Balgach, Switzerland). The amplification reactions were performed in 25µl volume, containing 20 mmol/L tris-HCl (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 µmol/L of dNTPs, 1µM/L of each primer, 30 ng of genomic DNA and 1.5 U of Taq DNA polymerase (Life Technologies, Basel, Switzerland). Denaturation was performed at 95 °C for 5 minutes, followed by 35 cycles comprising denaturation at 94 °C for 30 s, primer annealing at 36 °C for 30 seconds and extension at 72 °C for 60 s. A final extension was carried out at 72 °C for 7 minutes to ensure fragment elongation. The amplification products were separated by gel electrophoresis on precast 6 % poly(NAT) gels (Elchrom Scientific, Cham, Switzerland) visualized as described above. RAPD patterns were converted in two dimensional binary matrixes and analyzed using NTSYS-pc package. For each pair of strains, a simple matching (Sm) coefficient was calculated, and a UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

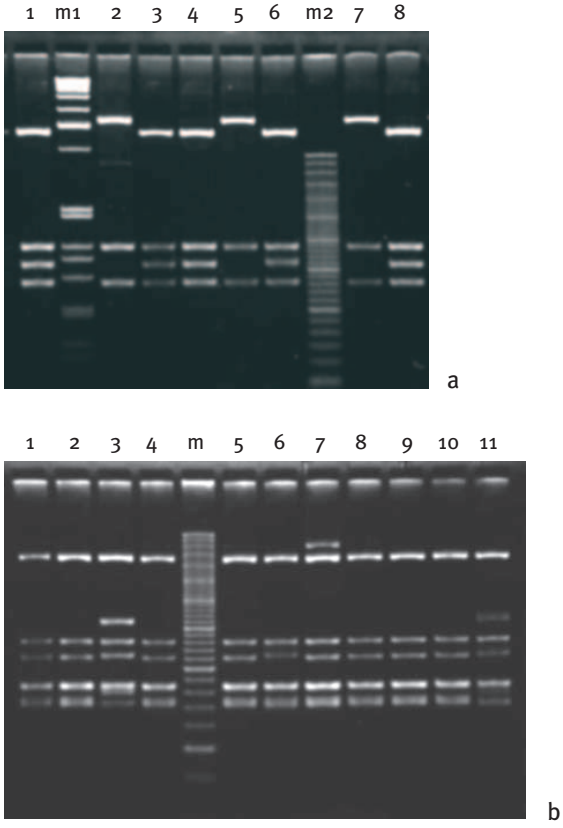

Figure 1.
Restriction analysis patterns of 18S rDNA + ITS1 amplicons using *Msp*I from representatives of the indigenous *Saccharomyces sensu stricto* isolates (a) (line 1: H7; 2: H6; 3: H5; 4: H4; 5: type strain *S. paradoxus* CBS 432; 6: H3; 7: type strain *S. paradoxus* CBS 432; 8: type strain *S. cerevisiae* CBS 1171; m1: 1 kb ladder; m2: 100 bp ladder) and *Hae*III (b) (line1: H1; 2: D1; 3:H2; 4:D2; 5: H3; 6:D3; 7:H4; 8: D4; 9: H6; 10: type strain *S. paradoxus* CBS 432; 11: type strain *S. bayanus* DBVPG 6171 (CBS 380); m: 100 bp ladder

Results

A total of 40 yeast strains were isolated from different wine fermentation phases (Table 1). The data obtained by physiological and morphological tests allowed grouping the isolates into the *Saccharomyces sensu stricto* group. Isolates that were physiologically confirmed were further differentiated by molecular methods. The 18S rDNA+ITS1 of 40 isolates and four reference strains were amplified with universal primer pairs NS1/ITS2. The PCR products were digested with restriction endonucleases *Msp*I and *Hae*III separately. The species-specific restriction patterns were obtained and compared to corresponding reference strains (Fig. 1)

When the rDNA gene region was digested with *Msp*I all isolates from both investigated locations obtained characteristic patterns for species *S. cerevisiae* except isolate H6, from location Sv. Nedjelja, which exhibited characteristic pattern for *S. paradoxus*. Additionally, isolate was physiologically confirmed by growing on D-mannitol as sole carbon source. After digestion with *Hae*III, two isolates, H2 and H8 generated characteristic patterns for *S.bayanus/S. pastorianus*. However, these isolates were not in agreement with the result obtained by physiological tests, such as growth at 37 °C in vitamin free media. Our results demonstrate that these tests are very often inappropriate for delineation of sibling species within *Saccharomyces sensu stricto* group White (1990). All other isolates from both investigated locations exhibited characteristic molecular pattern for *S. cerevisiae/S. paradoxus*.

Isolates tested through PCR-RFLP were further analyzed by RAPD in order to identify single strains. The four dendrograms were constructed showing genetic diversity of isolates within particular phase of spontaneous alcoholic

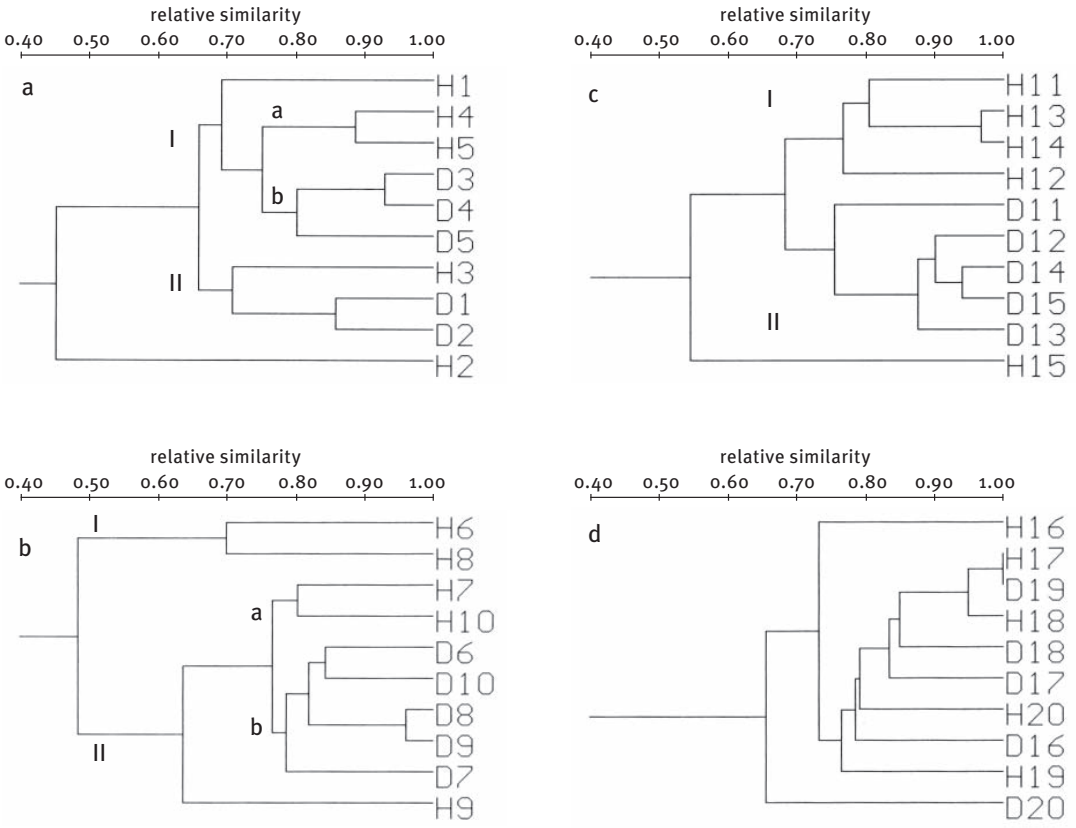

Figure 2.
Dendrograms of *Saccharomyces sensu stricto* strains derived by RAPD fingerprints using four different 10-mer primers within phase I (a), phase II (b), phase III (c), phase IV (d)

fermentation. RAPD method yielded characteristic DNA patterns for each tested strain. After gel electrophoresis, a different number of polymorphic bands was determined dipending on the primers used. Some primers resulted in very similar patterns whereas some primers resulted in more informative ones.

The dendrogram derived from RAPD profiles (Fig. 2a) shows that all strains isolated from initial stage of fermentation are grouped in two major clusters except single strain H2. Strain H2 significantly differed from all other tested strains. It belonged to *S. bayanus* species. The first cluster could be divided in two subgroups at similarity level of 0.70. Majority of strains from location Dingač belonged to subgroup I b. Strains D3 and D4 are very close related and also strains H4 and H5 from Sv. Nedjelja location.

The dendrogram (Fig. 2b) shows that isolates from second (vigorous) fermentation phase are grouped in two major clusters at similarity level of 0.50. The first major cluster comprised two isolates belonging to *S. paradoxus* (H6) and *S. bayanus*/*S. pastorianus* (H8) while all other isolates were grouped within second major cluster. The second cluster, that comprises about 80% of all strains analyzed, could be further divided into two subgroups. All strains from location Dingač were grouped within subgroup IIb whereas two strains D8 and D9 were almost identical (similarity level 0.95).

Strains from third fermentation phase clearly show grouping depending upon particular location (Fig. 2c). Strains were grouped in two major clusters. Majority of strains from location Sv. Nedjelja were grouped within cluster I while all strains from location Dingač were grouped within cluster II. Isolate H15 had a unique RAPD profile and that single isolate diverged at similarity level of 0.55 from all other tested strains.

According to the resulting dendrogram in Fig. 2d strains from the last fermentation phase were not grouped in clusters. Isolates were grouped in small subgroups comprised of very closely related strains whereas two strains isolated from different locations were found to be identical (H17 and D19). Generally, advancing the process strains became closer related.

Discussion

Since classical methods for identification and characterization of yeasts, based on morphological, physiological and biochemical characteristic require considerable experience in the evaluation of the tests, additional molecular characterization is needed (Redžepović et al., 2002). For accurate identification at the species level PCR-RFLP of 18S rDNA + ITS1 was used, while RAPD fingerprinting was used for strain differentiation. The latter methods analyze the genome of the yeasts and have several advantages, they are rapid and reproductive. Molecular techniques are already widely applied for typing yeasts in the fermentation processes. However, conventional identification methods were found rather difficult because of high level of phenotypic variability among isolates.

The restriction analysis of the PCR-RFLP of 18S rDNA + ITS1 resulted in characteristic and reproducible band patterns which could be clearly attached to the appropriate species within *Saccharomyces sensu stricto* group, specially comparing them to corresponding type strains. Identification by using PCR-RFLP analyses confirmed that *S. cerevisiae* was dominant species during spontaneous fermentation of cv. Plavac mali must, despite the incidence of one *S. paradoxus* (isolate H 6) and two *S. bayanus*/*S. pastorianus* (isolates H2 and H8) at the location Sv. Nedjelja. However, due to their ecological and enological importance their existence should not be discharged. Moreover, considering previous results obtained by Redžepović et al. (2002) which described large population of *S. paradoxus* isolated from the grape growing region in the north-western part of Croatia highlighted the incidence of *S. paradoxus* in niche habitats of Croatian vineyards. A number of previous studies (Naumov, 1996; Naumov et al., 1992; 1998) have suggested that *S. paradoxus* is usually found in oak exudates, bark, uncultivated soils and insects and little has been known as a species that can be present in man-made environment. Although *S. cerevisiae* and *S. bayanus* are widely regarded as the principle yeasts of wine fermentation with the most efficient fermentative catabolism, investigations confirmed positive enological characteristic of *S. paradoxus* in wine biodeacidification (Redžepović et al., 2003). *S. paradoxus* produces wines with low residual sugar and sensory profile different from that produced by *S. cerevisiae* (Majdak et al., 2002).

Results obtained by RAPD method confirmed applicability and sensitivity of this method for identification of *S. cerevisiae* strains. RAPD analysis may be valuable for strain typing (Baleiras Couto et al., 1995; Molnar et al., 1995; Queasada et al., 1995; Xufre et al., 2000). Results suggest that it may be also useful for identification of related species within the *Saccharomyces sensu stricto* group (Fernández-Espinar et al., 2003). It has been shown in many ecological studies that succession of different strains is involved in the course of spontaneous alcoholic fermentation (Versavaud et al., 1995; Nadal et al., 1996). Comparison of strains within each fermentation phase revealed differentiation among strains from different locations but also variation among strains sharing the same investigated area. Although edapho-climatic conditions of two investigated locations are almost the same, each vineyard characterized by specific microclimate resulted in the particular *S. cerevisiae* microflora (Cavalieri et al., 1998). Considerable level of similarity was detected within each locality. Those strains were grouped mostly in same clusters, particularly strains from Dingač location. At the end of the process, it was not possible to determine any rule that specified diversity among strains with the exception of two identical strains H17 and D19 from different locations. Generally, it can be observed that as the fermentation advances, strains of both investigated areas become closely related. It could be explained with stress conditions related to higher ethanol concentrations at this final stage which results in a stronger selection of similar strains. Considering results, none of the isolated strains prevailed.

The RAPD fingerprints of all isolates obtained from each location were also compared (data not shown). These results indicate coexistence of strains with no clear predominance throughout the whole fermentation process on each investigated locality, and consequently, strains are simultaneously active.

Conclusions

The results of this study showed that the RAPD assay is able to separate species within the *Saccharomyces sensu stricto* group. However, in order to confirm this statement, further investigation is needed. Different *S. cerevisiae* strains could be used to enhance the flavor profile of wines from given regions. Results of these investigations will enable further selection of the most efficient yeast strains and their application as a starter culture in wine biotechnology. It remains to be investigated in further selection program whether the indigenous yeast microflora of region can contribute to the production of characteristic wines and expression of varietal specificities.

References

Angelo K.J., Siebert A. (1987). New Medium For Detection of Wild Yeast in Brewing Culture Yeast. J. Amer. Soc. Brew. Chem. 45: 135-140.

Baleiras Couto M.M., Vogels J.T., Hofstra H., Huis J.H., Van der Vossen J.M. (1995). Random amplified polymorphic DNA and restriction enzyme analysis of PCR amplified rDNA in taxonomy: two identification techniques for food-borne yeasts. J. Appl. Bacteriol. 79 525-535.

Cavalieri D., Barbiero C., Casalone E., Pinzauti F., Sebastiani F., Mortimer R., Polsinelli M. (1998). Genetic and Molecular Diversity in *Saccharomyces cerevisiae* Natural Population. Food Technol. Biotechnol. 36: 45-50.

Dlauchy D., Tornai-Lehoczki J., Peter G. (1999). Restriction enzyme analysis of PCR amplified rDNA as a taxonomic tool in yeast identification. Syst. Appl. Microbiol. 22: 445-53.

Fernández-Espinar M.T., Barrio E., Querol A. (2003). Analysis of the genetic variability in the species of the *Saccharomyces sensu stricto* complex. Yeast, 20 1213-1226.

Fleet G.H. (2003).Yeast interactions and wine flavoure. Int. J. Food Microbiol. 86:11-22.

Majdak A., Herjavec S., Orlic S., Redzepovic S., Mirosevic N. (2002). Comparation of aroma coumpounds produced by *S .paradoxus* and *S. cerevisiae* strains. Food Technol. Biotechnol. 40: 103-109.

Molnar O., Messner R., Prillinger H., Stahl U., Slavikova E. (1995). Genotypic Identification of *Saccharomyces* Species using Random Amplified Polymorphic DNA Analysis. Syst. Appl. Microbiol. 18: 136-145.

Nadal D., Colomer B., Pina B. (1996). Molecular Polymorphism Distribution in Phenotyppically Distinct Populations of Wine Yeast Strains. Appl. Environ. Microbiol. 62: 1944-1950.

Naumov G.I., Naumova E.S., Korhola M. (1992). Genetic identification of natural *Saccharomyces sensu stricto* yeasts from Finland, Holland and Slovakia. Antonie van Leewenhoek. 61: 237-243.

Naumov G.I. (1996). Genetic identification of biological species in the *Saccharomyces sensu stricto* complex. J. Ind. Microbiol. 17: 295-302.

Naumov G.I., Naumova E.S., Sniegowski P.D. (1998). *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* are associated with exudates of North American oaks. Can. J. Microbiol. 44: 1045-1050.

Quesada M.P., Cenis J.L. (1995). Use of random amplified polymorphic DNA (RAPD-PCR) in the characterisation of wine yeasts. Am. J. Enol. Vitic. 46: 204-208.

Redžepović S., Orlić S., Sikora S., Majdak A., Pretorius I.S. (2002). Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. Lett. Appl. Microbiol. 35: 305-310.

Redžepović S., Orlić S., Majdak A., Kozina B., Volschenk H., Viljoen-Bloom M. (2003). Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. Int. J. Food Microbiol. 83: 49-61.

Romano P., Monteleone E., Paraggio M., Marchese R., Caporale G., Carlucci A. (1998). A methodological approach to the selection of *Saccharomyces cerevisiae* wine strains. Food Tech. Biotech. 36:69-74.

Schuller D., Valero E., Dequin S., Casal M. (2004). Survey of molecular methods for the typing of the wine yeast strains. FEMS Microbiol. Lett. 231:19-26.

Smole Možina S., Dlauchy D., Deak T., Raspor P. (1997). Identification of *Saccharomyces sensu stricto* and Torulaspora yeasts by PCR ribotyping.Lett. Appl. Microbiol. 24: 311-315.

Valente P., Gouveia F.C., Lemos G.A., Pimentel D., Van Elsas J., Mendonca-Hagler L.C., Hagler A.N. (1996). PCR amplification of the rDNA internal transcribed spacer region for differentiation of *Saccharomyces* cultures. FEMS Microbiol. Lett. 137: 253-256.

Vaughan-Martini A., Martini A. (1993). A taxonomic key for the genus Saccharomyces. Antonie van Leeuwenhoek. 16: 113-119.

Versavaud A., Courcoux P., Roulland C., Dulau L., Hallet J.N. (1995). Genetic Diversity and Geographical Distribution of Wild *Saccharomyces cerevisiae* Strains from the Wine-Producing Area of Charentes, France. Appl. Env. Microbiol. 61: 3521-3529.

White T.J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications. Academic Press, London, 315-322.

Xufre A., Simões F., Girio F., Clemente A., Amaral-Collaço T. (2000). Use of RAPD Analysis for Differentiaition among Six Enological *Saccharomyces* spp. Strains. Food Technol. Biotechnol. 38: 53-58.

acs73_14