

ISOLATION AND IDENTIFICATION OF LOCAL WINE YEAST STRAINS FROM DEALURILE BUJORULUI VINEYARD

Adrian GĂGEANU¹, Gheorghe CÂMPEANU¹, Camelia Diguță², Florentina MATEI³

¹U.S.A.M.V Bucharest, Faculty of Horticulture, Bd. Mărăști, nr. 59, Bucharest, Romania

²CBM Biotehgen, Bd. Mărăști, nr.59, Bucharest, Romania

³U.S.A.M.V Bucharest, Faculty of Biotechnology, Dep. Of Biotechnology, Bd. Mărăști, nr.59, Bucharest, Romania

Corresponding author e-mail: florentina.matei@biotehnologii.usamv.ro

Abstract

In the last years it has been given a high importance to promote on the market the Romanian wines with strong indigenous character related mainly to their sensorial profiles (aroma and taste). The wide use of commercial starter cultures, mainly applied to reduce the risk of spoilage and unpredictable changes of wine flavour, can ensure a balanced wine flavour, but it may also cause a loss of characteristic aroma and flavour determinants. This work belongs to a wider project in which, the goal is to demonstrate how the local yeast strains produce volatile compounds and fermentation metabolites which finally will modulate the variety characters on sensorial level. During 2009 -2010, from hundreds of yeast strains isolated from DealurileBujorului vineyard,9 yeast strains have been kept for their fermentative power. Classical and molecular identification of these strains have been performed. The classical identification has followed mainly the oenological practices and classification. The results show that these strains belong to the specie Saccharomyces cerevisiae under different subspecies groups (according to technological classification): S. ellipsoideus, S.bayanus, S.rosei, S.oviformis. By the use of molecular tools (ITS amplification), all the strains belongs undoubtedly to Saccharomyces cerevisiae group, but the intraspecific polymorphism is important. All the strains will be subject of fermentative and flavour development tests on local grapes variety.

Key words: Dealurile Bujorului vineyard, local yeast strains, Saccharomyces cerevisiae, autochthonous wine.

INTRODUCTION

It is well known that Romania is situated in top 10 wine producers in the world, with an average production of 5.5 mil hl/year. Almost 1 mil hl goes to the export. Taken into account that the Romanian wines have to compete with well know wines coming from other traditional wine-making countries (France, Italy, Spain, etc) or more recent producers as USA, Argentina or South Africa, there is a special need to find solutions to be competitive on the market. In the last years it has been given a high importance to promote on the market the Romanian wines with strong indigenous character related mainly to their sensorial profiles (aroma and taste) [3,8,9]. The distinctive flavour of wine depends on many variables as grape variety, viticultural practices, soil composition, which will influence the sensory descriptive analyses. In the same time

the influence of the local yeast strain should be taken into account [9, 10].

The wide use of commercial starter cultures, mainly applied to reduce the risk of spoilage and unpredictable changes of wine flavour, can ensure a balanced wine flavour, but it may also cause a loss of characteristic aroma and flavour determinants.

This work belongs to a wider project in which, the goal is to demonstrate how the local yeast strains produce volatile compounds and fermentation metabolites which finally will modulate the variety characters on sensorial level.

The work of valuable wine yeast isolation undergoes a few steps (isolation, purification, identification, laboratory testing and pilot-scale testing for their oenological characters). In this paper we will describe the identification step for strains isolated from a relative cool climate vineyard from Moldavia region, Romania.

Also, in the very last years, from different vineyard of colder climate (Northern Europe) have been isolated, aside strains of *S.cerevisiae*, different hybrids belonging to *S.uvarum*, *S. pastorianus* or *S.kudriavzevii* [4, 11, 12].

MATERIAL AND METHOD

The origin of the yeast isolation is situated in Dealurile Bujorului vineyard (Galati county), belonging to the Moldavian winemaking areal, specific for the production of white wines of high quality under a relative cooler climate.

Sampling and yeast isolation. The sampling has been performed during 2 harvest years, respectively 2009 and 2010. The grape berries have been harvested in September and October with pedicel. For each grape variety have been harvested samples from 3 points of the plot, from different levels of the rope: ground, middle and top.

The samples have been prepared in two ways. For the first series the berries have been harvested in aseptic conditions and introduced in an Erlenmeyer with sterile distillate water in order to collect the yeast from the grape surfaces. After 24 hours of shaking, from the suspension, plates with YEPD have been inoculated and cultivated during 48–72 hours at 27°C.

For the second series, the berries have been crushed in aseptic conditions and the natural fermentation has occurred. During the fermentation, three moments for sampling were chosen: beginning, middle and final phase of the fermentation. After applying Dörmér dilutions, the samples have been inoculated on YEPD.

Every isolated colony has been passed in slant on GYP medium and pure cultures have been obtained which were the identification subjects.

Yeast identification and characterization.

Two approaches have been followed for the identification: classical morphological and a molecular approach, by the use of ITS-RFLP.

In the case of classical identification there have been examined morphological and cultural aspects (shape and cell dimension of the cell) of each strain on liquid media after 3 days cultivation on GYP media at 27°C and on solid media YEPD; also, the pseudomycelia

formation on potato-agar media after 12 cultivation days have been observed.

For the molecular approach, the strains of oenological interest have been cultivated on GYP at 27°C during 24 hours/160rpm. The biomass has been collected and the DNA extraction has been performed according to an adapted method of Hoffman and Winston (1987) consisting of grinding the yeast with glass beads, phenol-chloroform extraction, and isopropanol precipitation [6]. The DNA has been conserved at -20°C until the PCR analysis.

For the PCR amplification has been used the following ITS primers:

ITS 1 (5'-TCCGTAGGTGAACCTGCGG -3')

and

ITS 4 (5'-TCCTCCGCTTATTGATATGC-3').

The amplification parameters have been the following: denaturation at 94°C/1.5 min; hybridization at 55°C/1.5 min; elongation at 72°C/2 min with a final elongation of 10 min.

Crude PCR products were digested with *Hinf*I, *Hae*III, *Hha*I (Promega) at 37°C during hours and the ITS sequences were determined from the digested fragments.

All the DNA products have been visualized by UV on gel electrophoresis run at 90 V during 60 minutes.

RESULTS AND DISCUSSIONS

During 2009 -2010, from hundreds of yeast strains isolated from Dealurile Bujorului vineyard (Galati county), 9 yeast strains (MD6 to MD14) have been kept for their oenological characteristics, respectively the fermentative power and alcoholic production (data not shown). Classical and molecular identification of these strains have been performed. The classical identification has followed mainly the oenological practices and classification.

According to these criteria, all the 9 strains belong to the specie *Saccharomyces cerevisiae* showing under the microscope around large globosetoellipsoidal yeast cells, size of 3-8 µm to 5-10 µm, with multilateral budding [1,7].

According to the technological criteria (old classification), including killer profile [8], these 9 strains belong to different subspecies groups: *S. ellipsoideus* (MD7, MD9, MD10),

S.bayanus(MD8, MD13), *S.rosei*(MD 11, MD 12), *S.oviformis* (MD6, MD 14).

The molecular characterization by PCR-ITS RFLP techniques was performed on the 5.8S-ITS region. The principle is to use a minimum number of restriction enzymes in order to obtain the maximum identification for strains, species or genders.

For the DNA extraction, the concentration and the purity of the DNA were adequate for all the strains (table 1).

Table 1. DNA quantification and purity

No.	Strain code	Conc. ng/ul	260/280	260/230
1.	MD6	176,7	1,55	0,48
2.	MD7	232,0	1,63	0,64
3.	MD8	249,4	1,68	0,74
4.	MD9	286,8	1,73	0,81
5.	MD10	113,5	1,38	0,35
6.	MD11	170,5	1,45	0,42
7.	MD12	213,3	1,55	0,53
8.	MD13	173,4	1,68	0,63
9.	MD14	105,7	1,35	0,33

The fragments obtained by *HhaI* digestion are shown in Fig. 1.

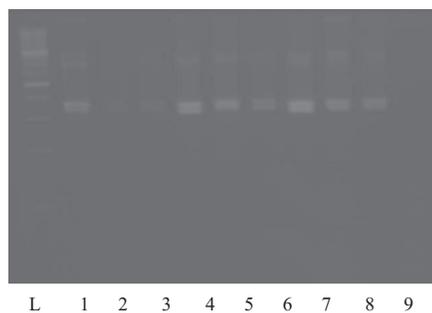


Fig.1. Fragments of yeast DNA after *HhaI* digestion (against 100 bp ladder) (MD 6 to MD9)

After *HhaI* digestion, all the 9 strains have shown two fragments of 385 bp, respectively of 365 bp and no difference could be noticed. Consequently, has been applied digestion with other restriction enzyme, respectively *Hae* III. After the digestion with *Hae* III (Fig. 2) all the strains showed two common fragments (of 320 bp and 230 bp). The difference appeared for three strains (MD6, MD11 and MD14) which have amplified a 165 bp fragment, while the rest of the strains had amplified two other fragments (180 bp and 150 bp). That was a

proof of an intraspecific polymorphism among the *S.cerevisiae* strains.

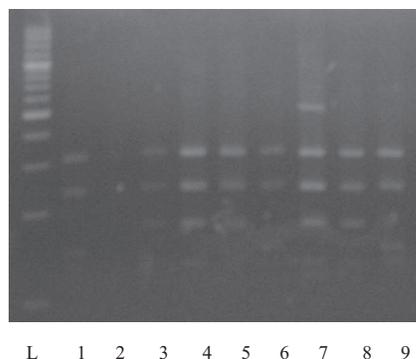


Fig.2. Fragments of yeast DNA after *Hae* III digestion (against 100 bp ladder) (MD 6 to MD9)

Regarding the *Hinf* I digestion, all the strains have showed the same fragments, respectively 365bp and 155 bp and no intraspecific aspects have been found (Fig. 3).

All the obtained fragments are summarized in Table 2.

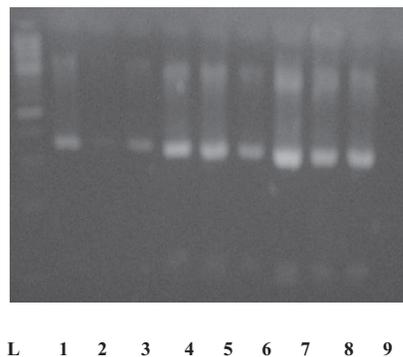


Fig.3. Fragments of yeast DNA after *HinfI* digestion (against 100 bp ladder) (MD 6 to MD9)

According to the results, all the strains belong to *Saccharomyces cerevisiae* group from a molecular point of view, while from technological point of view they have different species characteristics. It should be taken into account that some of the isolated strains may be hybrids belonging to *S.uvarums* or *S.kudriavzevii*, which should be proven by advanced molecular techniques (flow cytometry, microsatellite loci selection and analysis) [4,5,12]. These yeast hybrids may

appear by horizontal transfer event or by classical hybridization. According to Erny, 2012 both types of hybridization have taken place in the *Saccharomyces* genus.

Table 2. Fragments size (bp) obtained after digestion with different restriction enzymes of DNA yeast strains

No.	Yeast strain	<i>Hha</i> I (<i>Cfo</i> I)	<i>Hae</i> III	<i>Hin</i> fI
1.	MD6	385+365	320+230+165	365+155
2.	MD7	385+365	320+230+180+150	365+155
3.	MD8	385+365	320+230+180+150	365+155
4.	MD9	385+365	320+230+180+150	365+155
5.	MD10	385+365	320+230+180+150	365+155
6.	MD11	385+365	320+230+165	365+155
7.	MD12	385+365	320+230+180+150	365+155
8.	MD13	385+365	320+230+180+150	365+155
9.	MD14	385+365	320+230+165	365+155

As described in literature [5,11] these hybrids usually have been isolated from winery running in cold climate, known as cryophilic strains.

CONCLUSIONS

During 2009-2010 have been isolated hundred yeast strains from Dealurile Bujorului vineyard. According to the Hoffman and Winston (1987) protocol sufficient DNA quantities have been extracted from 9 yeast strains isolated from Dealurile Bujorului vineyard.

The identification has followed classical protocols (morphological characters), compared to the molecular characterization by PCR-ITS RFLP techniques.

The results show that these strains belong to the specie *Saccharomyces cerevisiae* under different subspecies groups (according to technological classification): *S. ellipsoideus*, *S. bayanus*, *S. rosei*, *S. oviformis*. By the use of molecular tools (ITS amplification), all the strains belongs undoubtedly to *Saccharomyces cerevisiae* group, but the intraspecific polymorphism high [1,2]. Moreover, it should be taken into account that some of the isolated strains are hybrids belonging to *S. pastorianus* or *S. kudriavzevii* [4], which should be proven by advanced molecular techniques (flow cytometry, microsatellite loci selection and analysis).

The adapted PCR-ITS RFLP employed method is a rapid and relative simple method to be used for the yeast isolated from vineyards and wineries. It can be used as a routinely, feasible and sensible method which can replace successfully the morphological identification for a high number of yeast strains in a short time.

We consider our identification work as a small but important step in the characterisation of our local wine yeast biodiversity as part of the world wide effort for the biodiversity conservation and valorisation.

Further, all these strains will be subject of fermentative and flavour development tests on local grapes variety.

REFERENCES

- [1]. Barnett, J. A., Payne, R. W. & Yarrow, D., 2001. *Yeasts: Characteristics and Identification*, 3rd Ed. Cambridge: Cambridge University Press.
- [2]. Bond U. 2009. *The genomes of lager yeasts*. Adv. Appl. Microbiol. 69:159–182.
- [3]. Burcea M. Radoi, F., 2005. *Cariotype analysis of some yeast strains isolated from Panciu wine region*. Rom. Biotech. Lett., 10 (2), p.2171 – 2177.
- [4]. Erny C, Raoult P, Alais A, Butterlin G, Delobel P, Matei Radoi F, Casaregola S, & Legras JL, 2012. *Ecological success of a group of Saccharomyces cerevisiae * Saccharomyces kudriavzevii hybrids in wine making environment*. App. Env. Microbiol. vol. 78 no. 9 3256-3265
- [5]. Gonçalves P, Valério E, Correia C, de Almeida JM, Sampaio JP. 2011. *Evidence for divergent evolution of growth temperature preference in sympatric Saccharomyces species*. PLoS One 6:e20739.
- [6]. Hoffman, C.S., and Winston, F., 1987. *A ten-minute preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli*. Gene 57, 267-272.
- [7]. Kurtzman, C. P. & Fell, J. W., 1998. *The Yeasts - a Taxonomic Study*, 4th edn. Amsterdam: Elsevier.
- [8]. Matei Florentina, Adrian Găgeanu, 2011. *Killer profile of wine yeast strains isolated in Dealurile Bujorului vineyard*. Rom Biotechnol. Lett., Vol. 16 (6), p.144-147.
- [9]. Matei Florentina, Brinduse Elena, Nicoale Getuta, Tudorache Aurelia, Razvan Teodorescu, 2011. *Yeast biodiversity evolution over decades in Dealu Mare-Valea Calugareasca vineyard*. Rom. Biotechnol. Lett., Vol. 16, No.1, Supplement, Bucharest, p.113-120.
- [10]. Pretorius I.S., 2000. *Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking*. Yeast 16:675-729.
- [11]. Sampaio JP, Gonçalves P. 2008. *Natural populations of Saccharomyces kudriavzevii in Portugal are associated with oak bark and are sympatric with S. cerevisiae and S. paradoxus*. Appl. Environ. Microbiol. 74:2144–2152.
- [12]. Schuller D., Alvesa H., Dequin S., Casal M., 2005. *Ecological survey of Saccharomyces cerevisiae strains from vineyards in the Vinho Verde Region of Portugal*, FEMS Microbiology Ecology 51, 167–177.