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# Wine-growing Areas in Oltenia (Romania) Major Natural Sources for the Isolation, Identification and Selection of Oenological Micoorganisms

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**Abstract**

The isolation and identification of the microbial agents responsible with the alcoholic fermentation process which cover all types of wine and the malolactic fermentation that is carried only by the red wines, begun almost when it was established the fact that the fermentations are biological processes done under the action of the enzymes that are produced by the microorganisms. As the passage of time, the searches of this kind have been boosted, so that almost no country is producing wines without having such concerns in the oenological microbiology field our researches also join on this line and have began with the isolation and identification of the yeast's present in different wine growing areas in Oltenia (Romania) then continuing with the isolation and identification of lactic bacteria present in these areas. During the study were isolated and identified yeast's strains of species belonging to *Saccharomyces ellipsoideus, Saccharomyces oviformis, Saccharomyces rosei, Metschikowia reukaufii, Pichia membranaefaciens, Candida vinaria, Kloeckera apiculata, Rhodotorula glutinis* and strains of the lactic bacteria that belong to the species: *Lactobacillus plantarum, Leuconostoc mesenteroides, Oenococcus oeni*. Knowing the physiologic and biochemical characteristics of these organisms, we can determine how they can influence positive or negative quality of the wine, the research will be continued to determine what types of relationships are established between these types of microorganisms both in the vineyard area and in wine.

***Keywords:*** vineyard area, yeast's, lactic bacteria, isolation

## Introduction

Over the last 3-4 decades oenological microbiology has brought a fundamental contribution to improving the technologies of designing, wrapping and aging wine. Concerns in ecology yeast's and lactic bacteria, but also in using the techniques of biochemistry and genetics have raised arguments about the division between species and strains of the same species. It is justified, rightly, the need to investigate indigenous microbial flora, taking into account the characteristics of technology-specific metabolism, competition between bacteria and lactic yeast's issues with beneficial implications on the quality of wines (Marlat , 1992; Popa, 2008).

A wide variety of bacteriocins produced by lactic acid bacteria (LAB) has been reported from oenological environments (Navarro *et al.*, 2000; Rojo-Bezares *et al.*, 2007; Knoll *et al.*, 2008). Bacteriocins may play a role in the ecology of LAB during alcoholic and malolactic fermentations, and they could have interest as efficient antimicrobial agents to control spoilage microorganisms in wine making (Delves-Broughton *et al.*, 1996). *Lactobacillus plantarum* strains have been reported in grapes and at different stages of the wine making process (Navarro *et al.*, 2000; Knoll *et al.*, 2008), and this LAB species is highly versatile and able to survive in an ample range of ecosystems. *L. plantarum* regulatory systems are fully related to specific detection of environmental signals (Sturme *et al.*, 2007), competition with other bacteria and survival in a variety of environments, among which wine, with high acidity and ethanol content, presents some of the most stressing conditions for bacterial growth and survival.

The diversity of yeasts present on berries, phylloplane and in soil is one of the important factors responsible for wine fermentation (Sabate *et al.*, 2002; Fleet, 2003; Clemente-Jimenez *et al.*, 2005). Oenological yeast strains such as *Candida, Debaryomyces, Dekkera, Hanseniaspora, Hansenula, Issatchenkia, Pichia, Rhodotorula, Schizosaccharomyces and Zygosaccharomyces* have been well studied with regarding to the quality of wines (Ciani and Maccarelli, 1998; Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003). *Saccharomyces cerevisiae* is primarily used for fermentation of grape juice. Several studies report the low occurrence of *S. cerevisiae* among the natural yeast populations found on grape berries (Fleet, 2003). Renouf *et al.* (2005) reported that *Candida, Hansenula, Hanseniaspora, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula* and *Saccharomyces* were present on the surface of grape berries. *Dekkera bruxellensis/Brettanomyces* was often found to be associated

Tab. 1. Climatic conditions (average 50 years), types of soil in predominant and vocation orderfor the quality of wine centers took into study

| Vineyard center | N Latitude | Altitude (m) | Climatic conditions | | | | | Soil types | Oenoclimatic capacity (A) A = T + I - (P-250) |
|---|---|---|---|---|---|---|---|---|---|
| | | | annual | | during the vegetation period (1st of April to 30th of October ) | | | | |
| | | | The average temperature (°C) | Rainfall (mm) | Total temperature degrees (°C) | Hours of actual insolation | Rainfall (mm) | | |
| Tamburesti | 44°02' | 73 | 10.9 | 575 | 3353 | 1540 | 312 | Pseudo-glazed Psamosol | 4805 $A_3$ |
| Banu Maracine | 44°19' | 195 | 10.9 | 543 | 3403 | 1574 | 288 | Ruddy lap Preluvosoil | 4939 $A_3$ |
| Dragasani | 44°30' | 182 | 10.8 | 684 | 3316 | 1576 | 385 | Lap calcified Preluvosoil; Lap skeleton Preluvosoil Ruddy lap Preluvosoil Tipically FZ; Calcified Regosoil | 4754 $A_3$ |
| Targu Jiu | 45°02' | 210 | 10.4 | 816 | 3233 | 1450 | 433 | Clay FZ; Calcified Preluvosoil Tipically Luvosoil tipic on gravel | 4500 $A_2$ |

A = oenoclimatic ability ; T = total temperature degrees of vegetation period ; I = insolation during the growing season ; P = rainfall during the growing season ; A2 = wine area producing mainly red wines and secondary quality white wines ; A3 = wine producing area in the main wheel your aromatic wines and white wines of secondary quality

Tab. 2 . Number and percentage of isolated and identified yeast's strains from the different media and the species belong (viticultural centers Banu Maracine and Tamburesti, 2000-2008)

| Species of yeast's and the number of isolated and identified strains | Number of isolated and identified yeast's strains from the different media | | | | | | Percentage of the isolated and identified strains on the grapes variety (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Soil Tamburesti | Soil Banu Maracine | Grapes from the inferior part of stump | Grapes from the superior part of stump | Must in fermentation | Must at final fermentation | Rosioara (Tamburesti) | Riesling Italian (Banu Maracine) | Cabernet Sauvignon (Banu Maracine) |
| *Saccharomyces ellipsoideus (53)* | 4 | 9 | 13 | 8 | 19 | 25 | 15 | 42 | 43 |
| *Saccharomyces oviformis (29)* | 3 | 7 | 14 | 10 | 25 | 21 | 14 | 41 | 45 |
| *Saccharomyces rosei (15)* | 7 | 13 | 20 | 13 | 27 | 20 | 40 | 40 | 20 |
| *Metschnikowia reukaufii (1)* | - | - | 1 | - | - | - | 100 | - | - |
| *Pichia membranaefaciens (20)* | 15 | 10 | 25 | 15 | 15 | 15 | 65 | 20 | 15 |
| *Candida vinaria (12)* | 8 | - | 17 | 8 | 34 | 8 | - | - | - |
| *Kloeckera apiculata (28)* | 14 | 14 | 29 | 18 | 25 | - | 43 | 25 | 32 |
| *Rhodotorula glutinis (12)* | 25 | 33 | 42 | - | - | - | 50 | 25 | 25 |

Tab. 3. Number and percentage of isolated and identified yeast's strains from the different media and the species belong (viticultural centers Dragasani and Tamburesti, 2003-2007)

| No. Crt | Species | Dragasani | | | | | | | | Targu Jiu | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Media | | | | | | | | Mediu | | | | | | | |
| | | No strains isolated | Soil | | Grapes | | Must | | Species percentage | No. strains isolated | Soil | | Grapes | | Must | | Species percentage |
| | | | No. | % | No. | % | No. | % | | | Nr | % | Nr | % | Nr | % | |
| 1. | *Saccharomyces ellipsoideus* | 8 | 1 | 12.5 | 2 | 25 | 5 | 62.5 | 15.3 | 7 | 1 | 14.3 | 2 | 28.6 | 4 | 64 | 21.2 |
| 2. | *Saccharomyces oviformis* | 7 | - | - | 1 | 14.3 | 6 | 85.6 | 13.5 | 3 | - | - | 1 | 33.3 | 2 | 66.6 | 9.0 |
| 3. | *Saccharomyces rosei* | 4 | 1 | 25 | 1 | 25 | 2 | 50 | 7.7 | 2 | 1 | 50 | 1 | 50 | - | - | 6.0 |
| 4. | *Candida vini* | 4 | 2 | 50 | 1 | 25 | 1 | 25 | 7.7 | 5 | 2 | 40 | 3 | 60 | - | - | 15.1 |
| 5. | *Pichia membranaefaciens* | 6 | 2 | 33.3 | 2 | 33.3 | 2 | 32.2 | 11.5 | 2 | 1 | 50 | 1 | 50 | - | - | 3.0 |
| 6. | *Hansenula anomala* | 4 | 1 | 25 | 2 | 50 | 1 | 25 | 7.7 | 1 | - | - | 1 | 100 | - | - | 3.0 |
| 7. | *Kloeckera apiculata* | 4 | 1 | 25 | 2 | 50 | 1 | 25 | 7.7 | 7 | 2 | 28.6 | 3 | 42.6 | 2 | 28.6 | 21.2 |
| 8. | *Hanseniaspora uvarum* | 4 | 1 | 25 | 1 | 25 | 2 | 50 | 7.7 | 3 | 1 | 33.3 | 2 | 66.6 | - | - | 9.0 |
| 9. | *Rhodotorula glutinis* | 3 | 2 | 66.6 | 1 | 33.3 | - | - | 5.8 | - | - | - | - | - | - | - | - |
| 10 | *Metschnikowia pulcherima* | 1 | 1 | 100- | - | - | - | - | 1.9 | 1 | 100 | - | - | - | - | - | 3.0 |
| | Total | 52 | | | | | | | | 33 | | | | | | | |

with wineries and less commonly on grape berries (Ibeas *et al.*, 1996; Fugelsang, 1997; Martorell *et al.*, 2006).

Research undertaken by our research team over the last decade have been aimed at detecting natural sources where you can isolate, identify and select the micro organisms of oenological interest (yeast's and lactic bacteria).

**Materials and methods**

Vast vineyard area in which it holds Oltenia, we chose to study four center vines: Tamburesti, Banu Maracine, Dragasani and Targu Jiu, which have different climatic conditions, and vineyards are located on different soil types. In order to isolate, identify and establish the biochemical-physiological and the technological characteristics of yeast's and lactic bacteria, there have been used protocols approved and recommended by the OIV.

**Results and discussion**

*1. Geographical location, climatic conditions and soil quality vocation for wine-growing centers in the survey taken*

In Tab. 1. synthetic data are presented which outlines the geographical situation, but also pedoclimatic conditions of the wine centers taken in the study, on which ,in time it has been outlined the openoclimatic fitness.

Geographical position of the wine center took into study, determinate the type of temperate-continental climate with Mediterranean influences. Average annual temperature varies between 10.4 ° C in Targu Jiu and 10.9°C to Banu Maracine and Tamburesti . Average annual rainfall most consistent (816 mm) are in Targu Jiu and Dragasani (684 mm) and in the other two centers average annual rainfall is more restricted. Noteworthy is that in all four rainfall centers are not evenly distributed throughout the year, are more common in winter and spring.

To conduct physiological and biochemical processes in the cell are particularly interested in the climatic conditions present during the growing season (1st of April to 30th of October ), especially the period August to October when the final ripening of the grapes. It finds that the amount of degrees of temperature, hours of actual insolation and precipitation (as rain) offered during the growing season, ensuring the optimum deployment of the processes of growth and ripening of grapes.

It is reported that the Tamburesti, most rainfall in the period 1st of April to 1st of June and almost insignificant or missing during the period 1st of July to 1st of September , when processes such as biochemical and phisiological are known, if not involved with irrigation, to develop a hydro-thermal stress situation. Temperature at ground

Tab. 4. Physiological particularity and oenological aptitudes for the yeast's strain isolated and identified from the spontaneous micro flora (viticultural centers of Oltenia)

| Strains | Physiological particularity | | | | | | | | | | | | | | | | | Oenological aptitudes | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sugarrs fermentation | | | | | | Sugarrs asimilation | | | | | | Nitrate assimilation | Alcoohol, like only source of the carbon | Splitting of arbutine | Growth in the absence of vitamins | Growth in the presence of ciclohexamide | Optimal temperature for fermentation (°C) | Resistance at $SO_2$ total mg/l |
| | Glu-cose | Galac-tose | Zaha-rose | Mal-tose | Lac-tose | Rafi-nose | Glu-cose | Galac-tose | Zaha -rose | Mal-tose | Lac-tose | Rafi-nose | | | | | | | |
| *Saccharomyces ellipsoideus* S.E.B.M.30 | + | + | + | + | - | + 1/3 | + | + | + | + | - | + | - | - | - | - | - | 25 | 350 |
| *Saccharomyces oviformis* S.O.B.M.16 | + | - | + | + | - | + 1/3 | + | - | + | + | - | + | - | - | - | - | - | 18<br>25 | 350<br>150 |
| *Saccharomyces rosei* S.R.B.M.7 | + | - | + | - | - | + 1/3 | + | - | + | - | - | - | - | - | - | ± | ± | 18 | 100 |
| *Saccharomy-codes ludwigii* S. D.L.B.M. 1 | + | - | + | - | - | + 1/3 | + | - | + | - | - | - | - | - | - | - | - | 25-35 | > 350 |
| *Metschnikowia reukaufii* M.R.T.1 | + | - | - | - | - | - | + | - | - | - | - | - | - | + | + | - | - | 18<br>25-35 | 200<br>250-350 |

(+) = positiv (-) = negativ ± = poorly

Tab. 5 . Lactic bacteria isolated from the grapes, must and wine

| Medium | U.F.C/ml | Species to belong the strains identified | Number of the isolated strains | Percentage of the species in the medium |
|---|---|---|---|---|
| Grapes | $1x10^2 – 6x10^2$ | *Lactobacillus plnatarum* | 4 | 100 |
| Must | $1x10^4$ -$6x10^5$ | *Lactobacillus plntarum* | 1 | 5.66 |
| | | *Oenococcus oeni* | 4 | 20.66 |
| | | *Leuconostoc mesenteroides* | 10 | 73.68 |
| Must in alcohoolic fermentation | $4x10^2$-$5x10^2$ | *Leuconostoc oenos* | 10 | 100 |
| Wine in the malolactic fermentation time | $7x10^3$/ $8x10^7$ | *Leuconostoc oenos* | 23 | 100 |

level (all psamosoil) frequently reaches in July and August, during the day, 65 º C and the temperature difference between day and night sometimes 30-35°C

All types of soil in the studied areas, except pseudo-glazed psamosoil (Tamburesti) by their physical-chemical properties and grain size have a good microbiological activity. Based on extensive studies carried out (Teodorescu *et al.*, 1987) on correlations between climatic conditions during the period of vegetation, soil types and quality of wine has outlined an indicator called synthetic oenoclimatic fitness (Tab.1.). Shared values of this indicator wines centers on vocation for quality into two categories: A2 - producing mainly for white wines and red wines secondary (Targu Jiu) and A3 - producing mainly red wine and aromatic secondary and white wine quality (Banu Maracine, and sometimes Dragasani and Tamburesti).

*2. Structure and microbial quality of architecture (yeast's and lactic bacteria)*

Yeast's of strains isolated and identified Tamburesti and Banu Maracine wine centers (Tab. 2.) belong to the species*: Saccharomyces ellipsoideus, Saccharomyces oviformis, Saccharomyces rosei, Metschnikowia reukaufii, Pichia membranaefaciens, Kloeckera apiculata, Rhodotorula glutinis.*

It finds a wide range of conveerului yeast's, especially predominantly sporogenes species. Isolated and identified strains were present in the soil, on the grapes and in the must of course. The only strain, belonging to the species *Metschnikowia reukaufii* was isolated and identified only by placing the grapes in the bottom of the logs at the only *Roşioară* variety , which is specific to the Tamburesti sandy area.

Share isolated and identified strains of different grape varieties shows that it doesn't matter the colour of grains, but the area where are planted the grape varieties. So, for example, Tamburesti especially predominant strains of the species: *Pichia membranaefaciens, Kloeckera apiculata* and *Rhodotorula glutinis*, when the Banu Maracine *Saccharomyces ellipsoideus* predominant species, *Saccharomyces ellipsoideus, Saccharomyces oviformis* and *Saccharomyces rosei*.

Results from the other two wine centers, Dragasani- and Targu Jiu (Tab. 3.) confirms the existence of a wide range of species of yeast's. And in these two wine centers, most strains of isolated and identified yeast's were found in grapes and must, it is true that in these centers we can not talk about a particular share of sporogen yeast's, further more as the vineyard is located more to the North at an higher altitude and a more consistent rainfall. Regarding yeast's spontaneous microflora present in the four wine centers, is sufficiently rich in species of oenological interest (especially in Banu Maracine, Tamburesti and Dragasani), as an important source of selection of the best-performing strains with which to finish fermentation of sugars and to get under controlled conditions, aromatic red and white wines of a certain quality.

We show, for example, skills and physiological particularities of oenological strains of isolated and identified yeast's from spontaneous flora, which are present in wine in Oltenia (Tab. 4.).

From the presentation of the physiological particularities and oenological skills of their strains of selected yeast's, we can easily find that many of them deserve to be used in technological processes of drafting great wines.

Distribution and frequency of lactic bacteria taken into study (grape must, wine) are different from one year to another, depending on climatic conditions offered by the vineyard and state of health of the grapes (Tab. 5.).

On the grapes, in must and wine there has been found lactic bacteria belonging to the species *Lactobacillus plantarum, Leuconostoc mesenteroides* and Oenococcus *oeni; Lactobacillus plantarum* was present in grapes and must, *Leuconostoc mesenteroides* in must and *Oenococus oeni* only in wine.

**Conclusions**

The four wine areas, taken in the study of Oltenia: Tâmbureşti, Banu Maracine, Drăgăşani, Targu Jiu, have ecopedoclimatic conditions that enables to obtain red and aromatic white wine of a high quality, a good part of them being among the wines that can carry a designation of origin.Architecture of the yeast's present in wine areas of Oltenia has a complex structure, predominantly the

ones that take part of the sporogene yeast's species , most of them present in soils and grapes, hence go to wines and musts. Structure and quality of the architecture yeast's is primarily dependent of the ecopedologic conditions from each vineyard.

Distribution and frequency of lactic bacteria and their performance are very different depending on the environment (grape must, wine). The frequency of lactic bacteria on the grapes is more restricted than the one of the yeast's.

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