RESEARCHS REGARDING TAXONOMIC IDENTIFICATION OF SPONTANEOUS MICROFLORA FROM VITI-VINICOL CERNAVODA CENTRE FROM MURFATLAR VINEYARD

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Abstract: *Wine microbiology researchers studies local microflora in order to selection of yeast strains, providing through controlled fermentation, high quality wines. It put thus, the theoretical basis of driven fermentation from biological point of view. Knowing the main species of yeasts of the vineyard, their morphological, physiological and oenological characteristics, the relationship between them can easily occur in the alcoholic fermentation of must and can be easily prevented many " wine accidents." The present study from the viti-vinicol Cernavoda Centre, from Murfatlar vineyard conducted in 2009-2011 period, has the aims:- isolation and identification of wine yeast species existing in spontaneous microflora;- morpho-physiological and biochemical,charac-terization and their taxonomic classification. This study will determine the succession of yeasts species during alcoholic fermentation and the proportion they have in different stages of vinification. The pure cultures obtained and analyzed will represented a data base for the future researches. Finally, were isolated 183 yeasts strains from the grapes, unfermented musts and musts in different stages of fermentation. The methods of sampling, isolation, culture and clasification used in researches, were the classics and described by Domerq (1956), Lindner (1913), Lodder (1970), Kreger van Rij (1984) and Kurtzman and Fell (2006). According to the international classification these 183 yeasts strains belong to two classes: Ascomycetes with three genres (Saccharomyces, Hansenula, Pichia) and Deuteromycetes with four genres (Candida, Kloeckera, Rhodotorula and Torulopsis). During alcoholic fermentation, the microorganisms present in musts, develops in a certain succession, which is conditional by the relationships that are established between different groups and were identified species that start fermentation, fermentation leading species and species present only at the end of alcoholic fermentation, called "species for wine finishing". Succession of yeasts species is influenced by increasing temperature and alcoholic degree and the degree and intensity of sulfur administration.*

Key words*: yeasts species, alcoholic fermentation, yeasts isolation*

INTRODUCTION

The preoccupations of Romanian researchers in the field of wine microbiology are directed to identifying and classifying species of yeasts from different vineyards in the country, and to the selection of yeast strains with superior attributes for obtaining quality wines.

Influence of selected yeasts on wine quality was and is still a subject of research. It was shown that the best results are obtained when the selected yeast is isolated from spontaneous microflora of each vineyard, so that is imposing the need to know the local microflora and its action on the wine quality, to control the fermentation process.

On the grapes surface in all stages of ripening, there are different microorganisms (yeasts, bacteria, fungi) that with the crushing and pressing operations will pass in the musts where due to the chemical composition of the musts (organic acids, sugars, minerals, nitrogenous substances, tannin and coloring substances) suffers a series of selections. Microflora yeast taxonomy was the subject of significant number of researches (J. Ribereau Gayon şi E. Peynoud- 1969; A. Kontek and Adriana Kontek- 1991; Fl. Danoaie-1989; G. Sandu-Ville-

1975). In the field of spontaneous microflora from the vineyards and wineries, the baseline study of is completed by Lodder and Lodder and Kreger van Rij.

The present study from the viti-vinicol Cernavoda Centre, from Murfatlar vineyard conducted in 2009-2011 period, has the aims:

- isolation and identification of wine yeast species existing in spontaneous microflora;
-morpho-physiological, and biochemical characterization and their taxonomic classification.

This study will determine the succession of yeasts species during alcoholic fermentation and the proportion they have in different stages of vinification. The pure cultures obtained and analyzed will represented a data base for the future researches.

MATERIAL AND METHODS

Following study were isolated, purified, characterized and identified a total of 183 yeasts strains, from the following ecological niches:

- the main grape varieties of the vineyard (Pinot gris, Riesling italian, Chardonnay, Fetească, Sauvignon, Pinot noir, Cabernet Sauvignon, Merlot);
- from unfermented musts;
- from musts at different stages of alcoholic fermentation (start fermentation; tumultuous fermentation, end of fermentation).

The isolation of yeast strains was performed from a single cell using the method of successive dilutions (Domercq 1956) and isolation in pure culture method (Lidner, 1913; Yarrow, 1998).

To identify and characterize the yeast strains were also pursued:

- cells-shape and size after cultivation for three days at 25 ° C, the liquid medium (grapes musts and Wickerham) and on the solid (must grapes with agarose gel);
- pseudomiceliu-formation (after cultivation for 12 days), in medium potato agarose gel;
- sporulation on synthetic medium-Gorodkova (comments after 30 days).

Physiological characteristics were determined using the following tests:

- fermentation of different sugars; - assimilation sugars; - nitrate-assimilation; - the using of ethyl alcohol as the unique carbon source; - split arbutin.

Taxonomic classification of the 183 strains of yeasts isolated and characterized, was made in conformity of: - Treaty taxonomy of Lodder 1970; - Changed name list of species of yeasts Barnett et al., 1983, Bandoni R.. J., 1984; - The third edition of the monography" The Yeasts Kreger van Rij" published 1984; - Monography" Yeasts, A Taxonomic Study" 5-th Revised and Enlarged Edition, published by Kurtzman and Fell., in 2006, which comprises about 60 genres and over 1500 species.

RESULTS AND DISCUSSIONS

According to the international classification these 183 yeasts strains belong to two classes: *Ascomycetes* with three genres (*Saccharomyces, Hansenula, Pichia*) and *Deuteromycetes* with four genres (*Candida, Kloeckera, Rhodotorula and Torulopsis*), and each genre has more species (table 1). The study allowed us to conclude that in spontaneous microflora existing species are found in different proportions to each other.

I noticed that yeasts are relatively rare on grapes and that their number is growing when the harvest is handled. But the microflora composition is changes in time, so that the dominant species on grapes, decrease their number during alcoholic fermentation process (table 1).

Table 1

Distribution of yeast microflora on genres and species according to ecological niches studied

Genre	Yeast specie	A*		B*		C*		D*		E*		A+B+C+D+E	%
		Nr. of strains	%	Nr. of strains	%	Nr. of strains	%	Nr. of strains	%	Nr. of strains	%		
Saccharomyces	Sacch. acidifaciens	-	-	-	-	-	-	-	-	2	1,09	**2**	**1,09**
	Sacch. bayanus	2	1,09	-	-	-	-	2	1,09	3	1,63	**7**	**3,82**
	Sacch. carlsbergensis	2	1,09	2	1,09	2	1,09	3	1,63	4	2,18	**13**	**7,10**
	Sacch. chevalieri	1	0,54	-	-	2	1,09	1	0,54	1	0,54	**5**	**2,73**
	Sacch. ellipsoideus	5	2,73	9	4,91	13	5,46	20	11,0	27	14,75	**74**	**40,5**
	Sacch. exiguus	-	-	-	-	2	1,09	-	-	-	-	**2**	**1,09**
	Sacch. heterogenicus	-	-	-	-	-	-	3	1,63	-	-	**3**	**1,63**
	Sacch. italicus	-	-	-	-	2	1,09	3	1,63	5	3,0	**10**	**5,46**
	Sacch. oviformis	2	1,09	-	-	2	1,09	4	2,18	6	3,27	**14**	**7,65**
	Sacch. rosei	-	-	-	-	2	1,09	3	1,63	5	3,0	**10**	**5,46**
Hansenula	Hansenula anomala	2	1,09	1	0,54	3	1,63	-	-	-	-	**6**	**3,27**
Pichia	Pichia fermentans	2	1,09	1	0,54	2	1,09	-	-	-	-	**5**	**2,73**
Candida	Candida mycoderma	3	1,63	1	0,54	3	1,63	1	0,54	-	-	**8**	**4,35**
Kloeckera	Kloeckera apiculata	10	5,46	-	-	10	5,46	-	-	-	-	**20**	**10,9**
Rhodoto-rula	Rhodotorula mucilaginosa	-	-	1	0,54	-	-	-	-	-	-	**1**	**0,54**
Torulop-sis	Torulopsis stellata	1	0,54	1	0,54	1	0,54	-	-	-	-	**3**	**1,63**
TOTAL:												**183**	**100**

* A= yeasts isolated from grapes; B= yeasts isolated from unfermented musts; C= yeasts isolated from musts at start of fermentation; D= yeasts isolated from musts in tumultuous alcoholic fermentation; E= yeasts isolated at end of fermentation.

The data from the table 1, it shown that the 183 yeasts strains, isolated and tested belonging to the species:

- *Saccharomyces acidifaciens*: 2 strains, isolated at end of fermentation process;
- *Saccharomyces bayanus*: 7 strains from which: 2 strains from the grapes, 2 strains from tumultuous fermentation and 3 strain, at the end of fermentation;
- *Saccharomyces carlsbergensis*: 13 strains from which: 2 strains from the grapes, 2 strains from unfermented musts, 2 strains from start of fermentation, 3 strains from tumultuous fermentation and 4 strains at the end of fermentation;

- *Saccharomyces chevalieri*: 5 strains from which: 1 strain from the grapes, 2 strains from start of fermentation, 1 strain from tumultuous fermentation and, 1 strain at the end of fermentation;
- *Saccharomyces ellipsoideus*: 74 strains from which: 5 strains from the grapes, 9 strains from unfermented musts, 13 strains from start of fermentation, 20 strains from tumultuous fermentation and, 27 strains at the end of fermentation;
- *Saccharomyces exiguus*: 2 strains, isolated from musts at start of fermentation;
- *Saccharomyces heterogenicus*: 3 strains isolated from musts tumultuous fermentation;
- *Saccharomyces italicus*: 10 strains from which: 2 strains from musts at start of fermentation, 3 strains from musts tumultuous fermentation, and, 5 strains at the end of fermentation;
-*Saccharomyces oviformis*: 14 strains, from which: 2 strains from the grapes, 2 strains from musts at start of fermentation, 4 strains from tumultuous fermentation and, 6 strains at end of fermentation;
- *Saccharomyces rosei*: 10 strains, from which: 2 strains from musts at start of fermentation, 3 strains from tumultuous fermentation and, 5 strains at end of fermentation;
-*Hansenula anomala*: 6 strains, from which: 2 strains from grapes, 1 strain from unfermented musts and, 3 strains from musts at start of fermentation;
- *Pichia fermentans*: 5 strains, from which: 2 strains from grapes, 1 strain from unfermented musts and, 2 strains from musts at start of fermentation;
- *Candida mycoderma*: 8 strains, from which: 3 strains from grapes, 1 strain from unfermented musts and, 3 strains from musts at start of fermentation and, 1 strain from tumultuous fermentation;
- *Kloeckera apiculata*: 20 strains, from which: 10 strains from the grapes and, 10 strains, from musts at start of fermentation;
- *Rhodotorula mucilaginosa*: 1 strain isolated from unfermented musts;
- Torulopsis stelata: 3 strains, from which: 1 strain from the grapes, 1 strain from musts at start of fermentation and, 1 strain from tumultuous fermentation.

In the oenological practice, only yeasts species involved in the alcoholic fermentation process are those what presenting importance. In figure 1, are presented proportional yeast species were isolated from musts in fermentation.

From figure 1, it can be seen that the largest share in the alcoholic fermentation, *Saccharomyces ellipsoideus* species holds - 46%, followed by species *Kloeckera apiculate* - 8%. Other species participating in the alcoholic fermentation in a smaller percentage in conformity with figure 1. These results obtained in viti-vinicol Cernavoda Centre, from Murfatlar vineyard, confirms research conducted in other vineyards and vine-growing centers in the world and in our country, which shows that in alcoholic fermentation, *Saccharomyces ellipsoideus* species participate in a proportion of 40-55% and, the start of alcoholic fermentation is given by *Kloeckera apiculata* species.

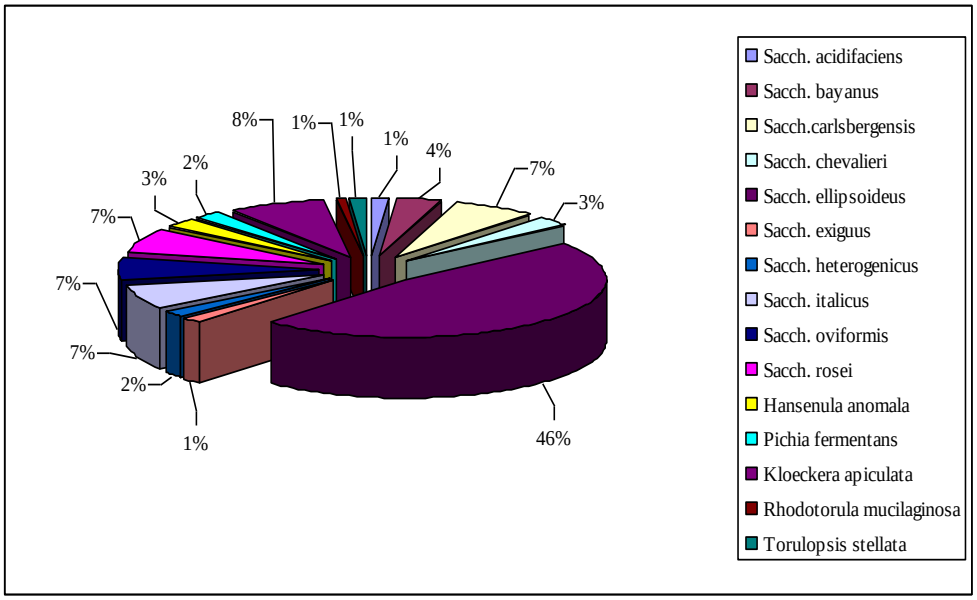

Fig. 1. The proportional presentation of yeast species isolated from musts in different stage of fermentation

During alcoholic fermentation process, the microorganisms present in musts, develops in a certain succession, which is conditional by the relationships that are established between different groups and were identified species that start fermentation, fermentation leading species and species present only at the end of alcoholic fermentation, called "species for wine finishing". Succession of yeasts species is influenced by increasing temperature and alcoholic degree and the degree and intensity of sulfur administration.

Spontaneous fermentation of the must is started by nesporogene species that provides beginning and the first phase of fermentation, of which predominate species is *Kloeckera apiculata* and in a smaller percentage *Torulopsis stellata*. Spontaneous fermentation is started of nesporogene species because they are on the surface of the grapes more than other species. How *Kloeckera apiculata* species can not tolerate only 4-5% vol alcohol, remains the species *Torulopsis stellata*, which shall operate until the medium reaches 7-8% vol alcohol. Sensitive to sulfur dioxide, the nesporogene yeast participation in the alcoholic fermentation is reduced, even when at vinification is applied normal sulphitation. Soon enough sporogene species and particularly *Saccharomyces ellipsoideus* species, dominates the medium so that at the end of fermentation, there is currently no unsporogene species (table 2).

The data in the table indicates the sequence of yeast species in the three stages of spontaneous fermentation process.

Table 2

Sequence of yeast species during alcoholic fermentation

Yeasts specie	Start of fermentation		Tumultous fermentation		End of fermentation	
	Nr. yeasts strains	%	Nr. yeasts strains	%	Nr. yeasts strains	%
Kloeckera apiculata	10	23,8	0	0,00	0	0,00
Torulopsis stellata	2	4,76	0	0,00	0	0,00
Total strains of unsporogene species	**12**	**28,56**	**0**	**0,00**	**0**	**0,00**
Saccharomyces ellipsoideus	13	30,95	20	51,28	27	50,94
Saccharomyces oviformis	2	4,76	4	10,25	6	11,32
Saccharomyces carlsbergensis	2	4,76	3	7,69	4	7,54
Saccharomyces chavalieri	2	4,76	1	2,56	1	1,88
Saccharomyces italicus	2	4,76	3	7,69	5	9,43
Saccharomyces rosei	2	4,76	3	7,69	5	9,43
Saccharomyces bayanus	0	0,00	2	5,12	3	5,66
Saccharomyces acidifaciens	0	0,00	0	0,00	2	3,77
Saccharomyces exiguus	2	4,76	0	0,00	0	0,00
Saccharomyces heterogenicus	0	0,00	3	7,69	0	0,00
Hansenula anomala	3	7,14	0	0,00	0	0,00
Pichia membranaefaciens	2	4,76	0	0,00	0	0,00
Total tulpini ale speciilor sporogene	**30**	**71,47**	**39**	**100,0**	**53**	**100,0**
TOTAL GENERAL	**42**	**100,0**	**39**	**100,0**	**53**	**100,0**

* The figures from the table indicate the frequency percentages for each of the three stages when samples were taken

The data presented in table 2, is noted that only 6 from those 14 species of yeasts, we find in the three moments of alcoholic fermentation.

Saccharomyces italicus species, whose percentage is low at the beginning of alcoholic fermentation, increases continuously during the course of it.

Other species, such as *Saccharomyces bayanus* and *Saccharomyces acidifaciens* appear during and at the end of alcoholic fermentation. These species called by some researchers "species for wine finishing", together with *Saccharomyces italicus* and *Saccharomyces oviformis* are those who due of their physiological properties, lead to obtaine dry wines, also and to some accidents of refermentation wines with residual sugar.

CONCLUSIONS

Were isolated and identificated 183 yeasts species from spontaneous microflora from vitivinicol Cernavoda Centre, situated in Murfatlar vineyard.

Taxonomic classification of these yeasts strains isolated and characterized, was made in conformity of: - Treaty taxonomy of Lodder 1970; - Changed name list of species of yeasts Barnett et al., 1983, Bandoni R.. J., 1984; - The third edition of the monography" The Yeasts

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According to the international classification these 183 yeasts strains belong to two classes: *Ascomycetes* with three genres (*Saccharomyces, Hansenula, Pichia*) and *Deuteromycetes* with four genres (*Candida, Kloeckera, Rhodotorula and Torulopsis*), and each genre has more species.

The predominant yeasts strains (number and percentage) belong to the following species: *Saccharomyces ellipsoideus* (74 strains), *Kloeckera apiculata* (20 strains), *Saccharomyces oviformis* (14 strains), *Saccharomyces carlsbergensis* (13 strains), *Saccharomyces italicus* (10 strains), *Saccharomyces rosei* (10 strains), *Saccharomyces bayanus* (7strains) and so on.

The study has determine the succession of yeasts species during alcoholic fermentation process and the proportion they have in different stages of vinification.

The pure cultures obtained and analyzed will represented a data base for the future researches and they will be used in obtaining of wines with typical attributes of the vine variety.

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