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"Developing Identity ON Yield, SOil and Site- DIONYSOS"

INTERREG V-A COOPERATION PROGRAMME: GREECE –BULGARIA 2014-2020"

REPORT

BY

INSTITUTE OF VITICULTURE AND ENOLOGY, PLEVEN

Activity 3.2. Selecting main local wine varieties and identifying their characteristics

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This report presents the scientific results of a research on the activity "Selecting main local wine varieties and identifying their characteristics", made by a research team of the Institute of Viticulture and Enology, Pleven, Bulgaria for the project DIONYSOS.The genetic identity of the varieties Dimyat, Misket Cherven, Mavroud, Pamid, Gamza, and Tamyanka have been confirmed by means of microsatellite analysis of grapevine samples. One sample was identified as clone of variety Misket Cherven and one sample was determined as an offspring of Mavroud.Grape mash from 10 grape batches from different regions of project area have been studied for presence microflora (yeast, bacteria) after spontaneous fermentation, and analyses have been made of the physicochemical characteristics of the resultant wines, along with sensory characterization. Thirty-eight morphological types, including 26 yeast strains and 12 bacterial strains, have been isolated and their morphological and cultural characteristics have been determined. The yeast strains have been checked for spore formation and sugar fermentation, and the strain variety of part of them has been determined using the molecular PCR method. The bacterial strains have been subjected to a number of studies for the purpose of their type and genus identification.Yeast cultures, differing in characteristics and properties, have been established, all of them having wine yeast specificity and high fermentation capacity. Strains similar or different in their DNA structure have been established in the individual micro regions. It can be expected that specific regional wines having suitable composition and sensory profiles can be produced using the local yeast microflora.

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Този документ е създаден в рамките на проект „Разработване на идентичност на добива, почвите и местностите"/ДИОНИСОС, Договор за субсидиране B2.6c.04/01.11.2017 който се осъществява с финансовата подкрепа на подкрепа на Програма за трансгранично сътрудничество ИНТЕРРЕГ V-A Гърция-България 2014-2020, съфинансирана от Европейския фонд за регионално развитие и от националните фондове на страните Гърция и България. Отговорността за съдържанието на документа се носи от Институт по лозарство и винарство-Плевен и при никакви обстоятелства не може да се счита, че този документ отразява официалното становище на Европейския съюз и Управляващия орган.

Introduction

Because of its ecological plasticity and high quality of the fruit, the vine is the most widespread culture in the world. As a result of the development of humanity, the selection of vine grape varieties from their natural habitats and their propagation and distribution, a large number of varieties are grown today, the main set of which is country-specific. In the conditions of dynamic development of viticulture, the work on selection and introduction of the varieties has expanded significantly, which leads to an increase in the number of varieties and distribution of the vine. The intensive movement of the varieties and the existence of a large number of their synonyms and homonyms in different countries are the cause of errors in the exchange of genetic plasm. In addition, as a result of the mutational variability of the vine, many varieties are characterized by significant intra-varietal diversity. The older the plantations, the more variations and clones can be found in their populations. Most of the old local and introduced varieties of vines are a mixture of valuable and a certain number of low-value plants with negative agrobiological characteristics. For greater accuracy, in addition to morphological features, the identification of the vine varieties common in different vineyards should be carried out by applying molecular markers. In the present work, different grapevine genotypes have been collected from South Bulgaria and more specifically from the regions of Haskovo and Kardzali.

All areas of the Dionysos project are located in the South-Bulgarian wine-growing region. The climate is transitional continental. The natural conditions in the southern region determine the specialization of viticulture in the wine production line, designed for quality and table red dry wines, as well as for the production of table grapes. In individual micro-regions there are conditions for the production of quality white wines and wines with a protected designation of origin.

Years ago, in Bulgaria under the influence of a number of political, climatic, anthropogenic and structural trends and changes it has been more noticeably observed reduction of the areas and loss of interest in growing vineyards planted with old local varieties. There is a very limited set of vine varieties with different production directionsin the South Bulgarian wine-growing region. There has been a trend of certain changes in the structure of the dessert and wine vine varieties in the last years.The favorable soil and climatic conditions of this region on the one hand, access to European pre-accession and then structural and

thematic funds on the other hand and the desire of wine companies in Bulgaria to change and diversify their products such as style, organoleptic characteristics and competitiveness on the third, led to creation of many new plantations with varieties of various origins and directions of use.The valuable local varieties have been preserved and a number of famous varieties of high quality have been introduced.

Established from millennia in the country practice in the wine sector, based on the vital importance of local wine grape varieties, both as a valuable genetic resource and a potential source of income and livelihood for the inhabitants of traditional wine-growingarea.The district of Haskovo is an established wine-growing micro-region in Bulgaria and viticulture in the Kardzhali region is small and fragmented, but are relied on as a source of income, especially in the lowland areas.

According to the guidelines of the ongoing European policy, the Bulgarian wine sector, part of which is wine production in Haskovo and Kardzhali, faces challenges to increase their competitiveness, preserving traditions and increasing social and environmental role in regional development aspects.

Under the growing competition on international markets Bulgaria should keep its place rediscovering the qualities of traditional local wine varieties. Under these conditions, the combination of nature and the potential of the varieties are a prerequisite for the production of wines of unique, individual character with which our country could achieve sustainable presence in the global wine market.

The aim of this studywas to select main local wine varieties and identifying their characteristics

To achieving that purpose next activities had to be carried out:

3.2.1. Analyses of the various local varieties in the areas of Haskovo and Kardzhali to identify the genuineness of the local traditional vine varietyes.

Material and Methods

Study Area: According to the provisions and the territorial division of the Regional Development Law, Haskovo and Kardzali are included in the South Central Planning Region. The relief of the area is too diverse. The two areas of research (Haskovo and Kardzhali) included in the Dionysos project are located in the Southern Balkan Xerothermal Soil Zone. The main and most widespread bioclimatic soil types in these areas are cinnamon forest soils, reedbeds and alluvial-meadow soils.

Southern geographical location of Haskovo and proximity to the Aegean Sea, which separate it from relatively lower slopes of the Eastern Rhodopes, as well as the easy penetration of warmer air in the valley of the Maritsa River, determine a transitional climate between the continental influence from the north and the Mediterranean from the south. Compared to the neighboring regions, the Mediterranean climate impact is more pronounced here. It is expressed mainly by higher annual average temperatures and the significant shifting of the main precipitation minimums and maximums. Winter is mild and short, summer - long and hot. The absolute value of the maximum temperatures is considered to be one of the most favorable in the country. The area is relatively windy. The region is characterized by moderate precipitation, with the snow cover from low to moderate. Kardzhali falls into the South-Bulgarian climatic region and more precisely in the East-Rhodopian climate region with warm Mediterranean influence. From the nature of atmospheric transferand transformation of air masses on the surface of the relief, is determined by the formation of trans-Continental and trans-Mediterranean climate. Winters are relatively mild and in the autumn-winter period, under the influence of Mediterranean cyclones drop some of the greatest rainfall (for more details please check the study of the regions 3.1.).

Sampling and processing of plant material

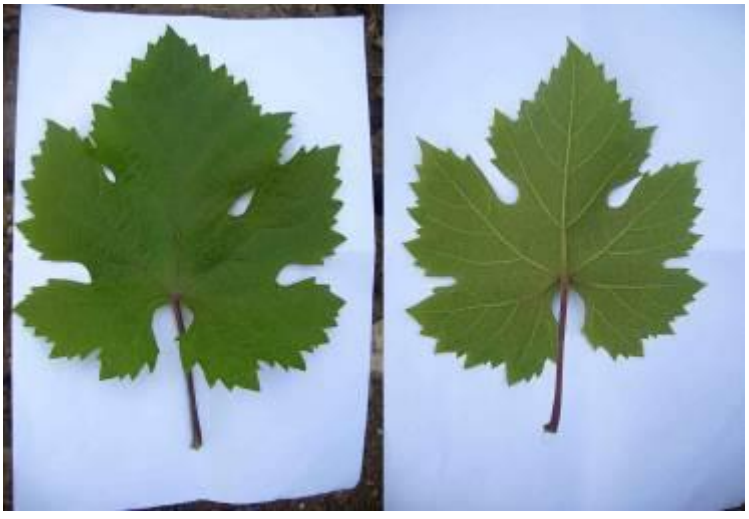
Leaf samples

Thirty five grapevine samples were collected from vineyards in six locations in South Bulgaria: village Dimitrovche (region Svilengrad), Lubimets (two locations), village Shishmanovo (region Harmanli), village Kolarovo (region Harmanli) and village Susam (region Haskovo). Grapevine samples belong to 6 old native Bulgarian cultivars: Dimyat, Misket

Cherven, Pamid, Mavroud, Gamza and Tamyanka(MAAF and BAFS, 2020). Young laves were collected and frozen at -70^{0}C.

Leaf of variety Dimyat from village Dimitrovche

Leaf of variety Misket cherven from village Dimitrovche



Leaf of variety Pamid village Kolarovo

Leaf of variety Tamyanka from village Shishmanovo

Leaf of variety Mavrud from village Shishmanovo

Leaf of variety Gamza from village Shishmanovo

DNA extraction

The extraction of plant DNA from the investigated grapevine samples has been performed with Qiagen_DNeasy 96 Plant Kit(Qiagen GmbH, Hilden, Germany).

Microsatellite analysis

Nine microsatellite markers adopted by the GrapeGen06(https://www1.montpellier.inra.fr/grapegen06/accueil.php) project forgenetic identification of grapevines have been used for microsatelliteanalysis of the grape cultivars: VVMD5, VVMD7,VVMD25, VVMD27, VVMD28, VVMD32(Bowers et al., 1996, 1999), VVS2(Thomas and Scott, 1993), ssrVrZag62and ssrVrZag79(Sefc et al., 1999). Polymerase chain reaction (PCR)conditions have beenset according to Dzhambazova et al.,2009. Forward primers of markers were labelled with6-FAM, ATTO565, ATTO550 and Yakima Yellow florescentdyes.Fragment analysis of the amplified products has been carriedout on an ABI 3130 Genetic Analyzer (Applied Biosystems,Foster city, CA, USA). For calculation of allelesizes, Orange 500 DNA Size Standard (500 bp) (MCLAB,San Francisco, CA, USA) has been used. Alleles were scoredusing the Genemapper v.4.0 software (AppliedBiosystems).

Construction of a dendrogram

Genetic distances between grapevine genotypes have been calculated as [-ln (proportion shared alleles)] using Microsat (Minch et al., 1997) and the obtained data have been used for the construction of a dendrogram using the programs KITCH from PHILIP package (Felsenstein, 1989) and Treeview (Page, 1996).

Table 1.

Sample №	Cultivar	Row	Num. of Plant	Origin
1	DIMYAT	2	2-1-3	Dimitrovche
2	DIMYAT	2	2-6-1	Dimitrovche
3	DIMYAT	2	2-10-3	Dimitrovche
4	MISKET CHERVEN	3	3-2-3	Dimitrovche
5	MISKET CHERVEN	3	3-5-2	Dimitrovche
6	MISKET CHERVEN	3	3-8-2	Dimitrovche
7	MAVROUD	6	6-5-2	Lubimets
8	MAVROUD	6	6-6-3	Lubimets
9	MAVROUD	6	6-9-4	Lubimets
10	PAMID	4	4-4-1	Lubimets
11	PAMID	4	4-6-1	Lubimets
12	PAMID	4	4-7-2	Lubimets
13	PAMID	7	7-4-1	Harmanli
16	GAMZA	11	11-4-4	Shishmanovo
18	GAMZA	11	11-7-5	Shishmanovo
19	GAMZA	11	11-8-4	Shishmanovo
20	TAMYANKA	21	21-4-4	Shishmanovo
21	TAMYANKA	21	21-7-2	Shishmanovo
22	TAMYANKA	20	20-7-2	Shishmanovo
23	TAMYANKA	22	22-6-1	Shishmanovo
24	MAVROUD	13	13-2-4	Shishmanovo
25	MAVROUD	13	13-3-4	Shishmanovo
26	MAVROUD	13	13-5-4	Shishmanovo
27	MAVROUD	13	13-7-4	Shishmanovo
28	MAVROUD	2	2-1-3	Susam
29	MAVROUD	2	2-3-1	Susam
30	MAVROUD	2	2-4-2	Susam
31	MAVROUD	2	2-5-2	Susam
32	TAMYANKA	18	18-3-2	Susam
33	TAMYANKA	18	18-4-2	Susam
34	TAMYANKA	18	18-5-4	Susam
35	TAMYANKA	18	18-7-1	Susam
36	PAMID	4	4-2-3	Susam
37	PAMID	5	5-4-1	Susam
38	PAMID	5	5-5-4	Susam

3.2.2. Isolation and identification of microorganisms (yeast, bacteria etc.) and identification and evaluation of their performance

Berry Sampling

Sample collecting

Dimyat

Misket Cherven

Mavrud

Pamid

Tamyanka

Gamza

Vinification process

1. Obtaining spontaneously fermented wines from 10 grape varieties in the Haskovo and Kardzhali regions.

We supplied to the **Biavin EOOD** company 10 grape samples in 5-6 kg quantities. The grape varieties and origin have been presented in Table 2.

Table 2. Experimental grape samples from the Haskovo and Kardzhali regions

Sample	Variety	Location
1	Dimyat	village of Dimitrovche , Svilengrad Municipality
2	Misket Cherven	village of Dimitrovche, Svilengrad Municipality
3	Mavrud	Lyubimets
4	Pamid	Lyubimets
5	Pamid	village of Kolarovo, Malkata Zvezda Cellar
6	Gamza	village of Shishmanovo, Bratanovi Cellar
7	Tamyanka	village of Shishmanovo, Bratanovi Cellar
8	Mavrud	village of Shishmanovo, Bratanovi Cellar
9	Mavrud	village of Susam, Starosel Cellar
10	Tamyanka	village of Susam, Starosel Cellar

The ten grape samples have been processed in thesame way: the grapes have been first destemmed into containers cleaned with spirt, then crushed and transferred into brand new

5 dm^3 PET containers. Each samplehas beenhomogenised and analysed for reducing sugars (the Schoorl chemical method), titratable acidity and pH(Yankov, 1992).

Sulphitation has been applied using a 5% solution of sulphurous acid in a 50 mg/dm^3 SO_2 dosage; then the samples have been placed at 25°C and the progress of the spontaneous alcoholic fermentationhas been monitored refractometrically (figure 1). The homogenisation of the solid parts with the liquid phase has been performed by stirring on a daily basis.

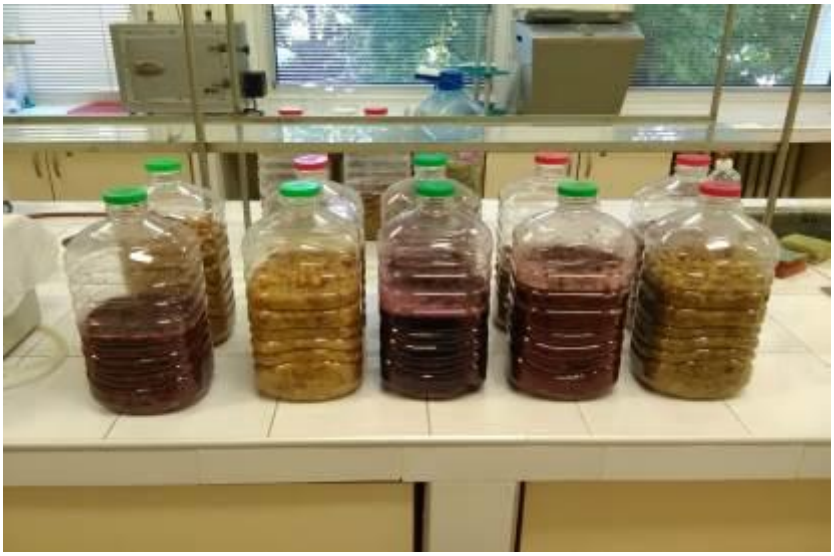


Figure 1. Spontaneous alcoholic fermentation of experimental grape samples

Figure 2. Experimental wines after separation of the solid parts

In all samples, the liquid phase has been separated from the solid parts without pressing; the wine has been placed in PET containers and stored for further investigation (figure 2).

One part of the experimental wines have been used for the isolation of available microorganisms, and another part for physicochemical analyses of certain indices.

An organoleptic analysis of the experimental wines has been made by a panel of 7 experienced oenologists.

Physicochemical and organoleptic profile of the spontaneously fermented experimental wines

The experimental wines obtained have been analysed according to the following indices: reducing sugars, alcohol, titratable acids, volatile acids and pH(Ivanov et al., 1979; Chobanova, 2007). The organoleptic characteristics of the experimental samples were determined by a tasting commission on a 100-point scale, by the indicators color, aroma, taste and general impressions (Tsvetanov, 2001) and by the method of the main characteristics (Prodanova, 2008).To accurately determine the differences between the wines from the branches of the respective variety, the average organoleptic indicators are depicted and presented with the help of graphical images in the form of spider diagrams (Bertrandand Kotseridis, 2004; Sieczkowski and Poinsaut, 2004).

Isolating morphological types of spontaneously fermented experimental wines.

Suitable dilutions in a sterile saline solutionhave been made, inoculated a malt extract agar complex culture medium (two repetitions) and a malt extract agar selective culture medium with 20 mg/dm^3 actidione added. The Petri dishes have been thermostated at 27^{o}C and the development of individual colonies have been monitored.

Pure cultures have been isolated from the morphological types established using Koch's method: at the 96th hour from the complex culture media, and at the 168th hour from the selective media. For the yeast strains, the isolation has been made in test tubes with 10 cm^3 of sterile grape juice in each; and for the bacteria, test tubes with 10 cm^3 of sterile bacterial medium have been used. The samples have been remained inthermostat at 25^{o}C for 5-7 days, and then they have been subjected to a number of tests. A collection of the isolated morphological types after growth has been placed in cold storage.

Morphological and cultural characterisation of newly isolated morphological types from spontaneously fermented experimental wines.

Morphological characterisation has been made of the isolated pure cultures of microorganisms. They have been identified as either yeast or bacteria. Their shapes, sizes, the situation of the cells in the medium and the vegetative propagation type have been determined. The morphological characterisation has been made on 72-hour liquid cultures, and the cultural characterisation on 72-96-hour colonies formed on grape juice – agar.

Results

Thirty five grapevine samples representing 6 old native Bulgarian cultivars were collected in 7 different location in Bulgaria. The group of collected grapevines consisted of 3 samples of DIMYAT, 3 samples of MISKET CHERVEN, 11 samples of MAVROUD, 7 samples of PAMID, 3 samples of GAMZA and 8 samples of TAMYANKA (Table 1). Genetic authenticity of these samples has been determined through microsatellite analysis with 9 microsatellite markers. The obtained microsatellite profiles of analyzed grapevine samples at 9 microsatellite markers are represented inTable3. Microsatelliteprofile of each sample has been compared with the profile of corresponding variety in VIVC (Vitis International Variety Catalogue(http://www.eu-vitis.de/index.php) and Bulgarian Vitis database (http://www.bulvitis-db.com/). The analysis of investigated 35 samples resulted in 8 unique microsatellite profiles. The genotypes of all grapevine samples from cultivars Dimyat (3 samples), Misket Cherven (3 samples), Gamza (3 samples) and Tamyanka (8 samples) have been found to be identical with those of the corresponding varieties. Ten out of eleven samples of Mavroud possessed genotypes identical with the genotype of variety Mavroud. Sample number 24 shared half of its alleles with Mavroud, which indicates first degree relationship with variety Mavroud. Six, out of 7 samples of PAMID are identical with variety Pamid. Sample number 10 has a microsatellite profile different from the profile of variety PAMID, but almost identical with a microsatellite profile of Misket Cherven, with the exception of two alleles at microsatellite locus ssrVrZAG79. Thus the sample number 10 has been considered as clone of varietyMisket Cherven.

The data from the microsatellite analysis of investigated 35 grapevine samples (Table 3) as well as microsatellite profiles of varieties Dimyat, Misket Cherven, Pamid, Mavroud, Gamza and Tamyanka and ten additional old native Bulgarian cultivars (Table 1) have been further

used for construction of a dendrogram. The obtained dendrogram demonstrate the genetic identity of investigated 35 samples, as well as the genetic relationship among them andwith other Bulgarian varieties (Fig. 3). Fifty one grapevine samples and varieties included in the dendrogramhave been distributed in 4 groups-A, B, C and D.The cultivar Varnenska Gamza remained outside the clusters, formed from the rest of the varieties, which defined it as a most divergent cultivar. The dendrogram show sixgroups of identical genotypes corresponding to the genotypes of cultivars Dimyat, Misket Cherven, Pamid, Mavroud, Gamza and Tamyanka. The first group located in cluster A consisted of 4 identical genotypes i.e. Gamza and samples 16, 28 and 19, which have beendetermined as Gamza by the microsatellite analysis. The second group located inside cluster B included 8grapevine samples (20, 21, 22, 23, 32, 33, 34 and 35) identical withcultivarTamyanka. This cluster included also two genetically related to Tamyanka varieties, Kravi Cici and Kozi Cici Cherveni.Cluster C contained two sub clusters of identical genotypes, one contains 3 samples (1, 2 and 3) identical with Dimyat and the second consisted of 11 samples (7, 8, 9, 25, 26, 27, 28, 29, 30 and 31 identical with Mavroud. According to the dendrogram, the Mavroud samples and sample 24 have been genetically close, which is in agreement with assumption that sample 24 is an offspring of Mavroud. Cluster C also contained cultivars Shefka, Shiroka melniska loza and Zarchin. Cluster D included two groups of identical genotypes related to Pamid (sample 11, 12 and13) and Misket Cherven (samples 4, 5 and 6). Closest relationship has been determined between Misket Cherven samples and sample 10, which has been identified through microsatellite analysis as a clone of Misket Cherven. Cluster D comprised also varieties Berbecel, Keratouda and Hora.The genetic identity of 33 out of 35 grapevine sampleshave been determined by means of microsatellite analysis.

Conclusion: The microsatellite analysis of 35 grapevine samples have been confirmed genetic identity of 33 out of 35 grapevine samples as follows: samples 1, 2 and 3 are identical with variety Dimyat, samples 4, 5 and 6 are identical with cultivar Misket Cherven, samples 7, 8, 9, 25, 26, 27, 28, 29, 30 and 31 are identical with variety Mavroud, sample 11, 12 and 13 are identical with variety Pamid, samples 16, 28 and 19 are identical with cultivar Gamza, samples 20, 21, 22, 23, 32, 33, 34 and 35 are identical withcultivarTamyanka

Sample 10 has been identified as clone of variety Misket Cherven. Sample 24 has been determined as an offspring of Mavroud.

Table 3. List of 35 grapevine samples of *Vitis vinifera* ssp. *sativa* L.,their microsatellite profile at 9 nuclear microsatellite loci and genetic identification of samples obtained through microsatellite analysis The names of cultivarsare spelled according to the VIVC. Allele sizes are given in bp.

Sample No	Analysed microsatellite loci																		Genotype
	VVS2		VVMD5		VVMD7		MVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		
1	143	143	242	248	239	249	249	255	180	182	234	246	250	264	188	204	237	259	DIMYAT
2	143	143	242	248	239	249	249	255	180	182	234	246	250	264	188	204	237	259	DIMYAT
3	143	143	242	248	239	249	249	255	180	182	234	246	250	264	188	204	237	259	DIMYAT
	143	143	242	248	239	249	249	255	180	182	234	246	250	264	188	204	237	259	Variety DIMYAT
4	135	143	234	242	249	253	255	255	184	195	236	258	256	256	188	194	259	259	MISKET CHERVEN
5	135	143	234	242	249	253	255	255	184	195	236	258	256	256	188	194	259	259	MISKET CHERVEN
6	135	143	234	242	249	253	255	255	184	195	236	258	256	256	188	194	259	259	MISKET CHERVEN
	135	143	234	242	249	253	255	255	184	195	236	258	256	256	188	194	259	259	Variety MISKET CHERVEN
7	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
8	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
9	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	Variety MAVROUD
10	135	143	nd	nd	249	253	255	255	184	195	236	258	256	256	188	194	249	257	Not determined [1]
11	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	PAMID
12	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	PAMID
13	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	PAMID
	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	Variety PAMID
16	133	135	228	228	247	255	239	255	186	195	228	260	272	272	188	204	249	249	GAMZA
18	133	135	228	228	247	255	239	255	186	195	228	260	272	272	188	204	249	249	GAMZA
19	133	135	228	228	247	255	239	255	186	195	228	260	272	272	188	204	249	249	GAMZA
	133	135	228	228	247	255	239	255	186	195	228	260	272	272	188	204	249	249	VarietyGAMZA
20	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA

Sample No	Analysed microsatellite loci																		Genotype
	VVS2		VVMD5		VVMD7		MVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		
21	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA
22	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA
23	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA
	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	Variety TAMYANKA
24	133	145	234	nd	243	249	241	249	180	186	218	260	240	256	188	194	237	239	Not determined [2]
25	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
26	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
27	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
28	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
29	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
30	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
31	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	MAVROUD
	133	145	234	242	239	249	239	241	180	182	258	260	256	272	188	194	237	243	Variety MAVROUD
32	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA
33	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA
34	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA
35	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	TAMYANKA
	133	133	230	238	233	249	241	249	180	195	246	268	264	272	186	196	251	255	Variety TAMYANKA
36	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	PAMID
37	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	PAMID
38	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	PAMID
	135	143	228	248	239	239	249	255	184	190	248	258	252	256	188	188	243	251	Variety PAMID

1- Not determined clone of MISKET CHERVEN

2- Not determined with parent MAVROUD

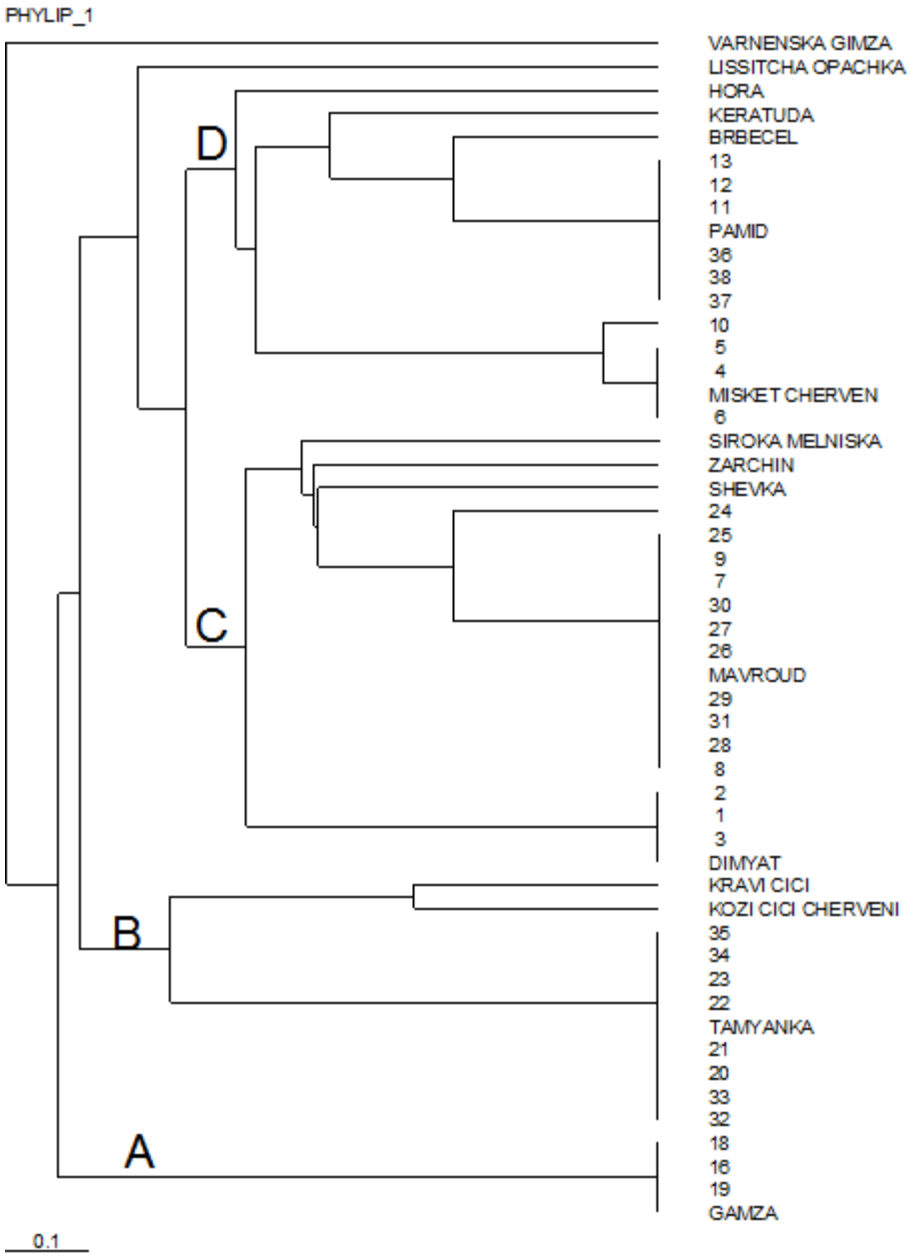


Fig. 3. Dendrogram of 35 grapevine samples and 16 old native Bulgarian varieties based on the data of 9 nuclear microsatellite markers. The varieties are grouped in 4 clusters (A-D).

3.2.2. Isolation and identification of microorganisms (yeast, bacteria etc.) and identification and evaluation of their performance

Berry Sampling

The results of sugars, titratable acidity and pH have been presented in Table 4.

Table 4. Physicochemical indices of experimental grape batches

Sample	Variety	Indices of experimental grape batches		
		Reducing sugars, [g/dm^3]	Titratable acids [g/dm^3]	pH
1	Dimyat	161.0	4.57	3.41
2	Misket Cherven	198.0	3.50	3.84
3	Mavrud	184.0	4.32	3.84
4	Pamid	210.0	3.71	3.99
5	Pamid	205.0	2.64	3.96
6	Gamza	189.0	4.97	3.68
7	Tamyanka	222.0	4.16	3.88
8	Mavrud	190.0	5.05	3.67
9	Mavrud	181.0	6.87	3.52
10	Tamyanka	236.0	5.49	3.90

The alcoholic fermentation dynamics is shown on fig. 2. Until the 24^{t}h – 36th hour, there has been no indication of a start of the alcoholic fermentation(figure 4). With most samples, the beginning of the process has been observed between the 48th and the 72th hour, and for some samples, for example Misket Cherven 2, Mavrud 9 and Tamyanka 10, the start of the alcoholic fermentation has been delayed by a further 24 – 48 hours. That has been probably due to the inhibiting effect of the sulphur dioxide added and the need for some time during which the available microorganisms could adapt to the environment. In the Tamyanka 10 sample, the osmotic shock on the yeast present in it may also have had certain influence. It is worth noting that the process in the Pamid 4 and 5 and Tamyanka 7 samples has been more dynamic regardless of the higher initial sugar content compared to the Dimyat 1 sample. On this basis, could be saidthere have been yeasts, differing in quantity, inhibitor resistance and fermentation activity on the grape samples. Some of their strains, after adaptation to the environment, could be expected to begin and conduct active alcoholic fermentation, demonstrating resistance to sulphur dioxide and osmotic pressure.

Most of the samples practically have been finished the alcoholic fermentation process on the 8-9th day. The dry matter concentrations according to the refractometer have been within 5-8% depending on the fermented sugars and the alcohol formed. A delayed and incomplete fermentation has been established with the Tamyanka 7 and Tamyanka 10 samples. This fact could be attributed to the low wine yeast concentration on the grapes and the lower yeast activity. The observation of the samples has ceased after the 13th day.

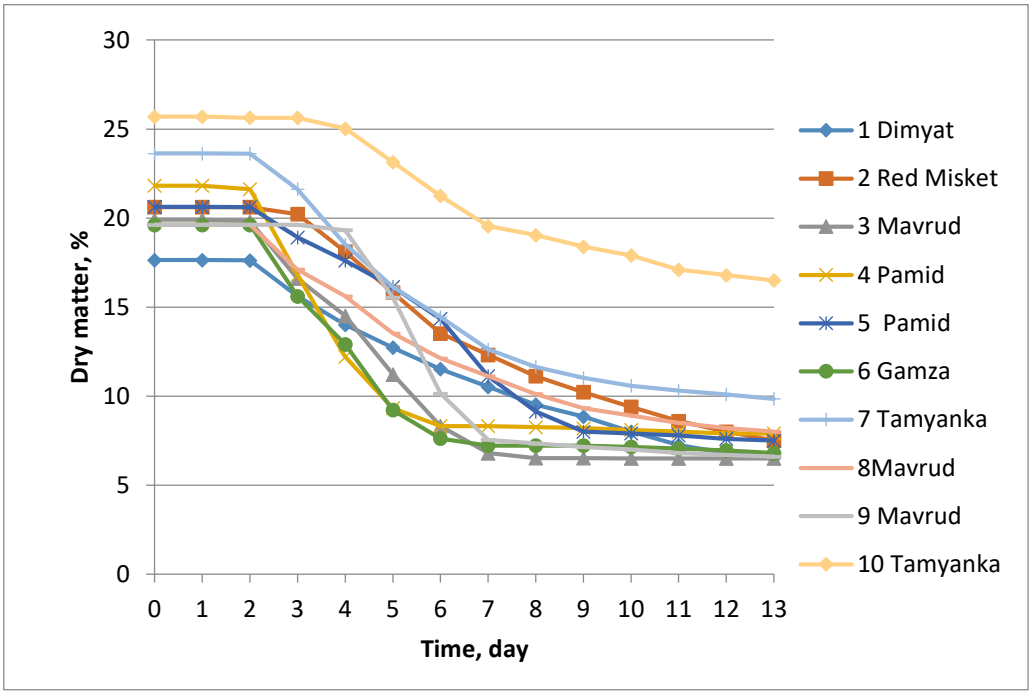


Figure 4. Alcoholic fermentation dynamics of experimental wines

Physicochemical and organoleptic profile of the spontaneously fermented experimental wines

The data from the analyses of the experimental wines (reducing sugars, alcohol, titratable acids, volatile acids and pH) are presented in Table 5.

The values of the reducing sugars correspond to dry wines in 8 of the 10 samples. That is in agreement with the fermentation completion degree controlled refractometrically. In the Tamyanka 7 and Tamyanka 10 samples, there are a lot of non-fermented sugars, their amount is particularly large in the latter.

The alcohol quantities formed correspond to the amount of fermented sugars, the mean conversion factor varying between 0.59 – 0.61. The highest alcohol content has been

established in the Tamyanka 7 and Pamid 4 samples. The alcohol content has been low in the Mavrud 8 and Mavrud 9 samples, and the lowest in the Tamyanka 10 sample where the process had not finished completely.

Table 5. Physicochemical composition of the spontaneously fermented experimental wines

№	Sample	Reducing sugars (g/dm^3)	Alcohol (%)	Titratable acids (g/dm^3)	Volatile acids (g/dm^3)	pH
1	Dimyat 1	1.28	10.3	6.67	0.36	3.29
2	Misket Cherven 2	1.68	12.0	4.64	0.47	3.76
3	Mavrud 3	1.68	11.2	7.93	0.27	3.56
4	Pamid 4	1.16	12.7	4.61	0.41	3.98
5	Pamid 5	1.16	12.4	4.38	0.36	3.86
6	Gamza 6	1.31	11.4	6.17	0.35	3.63
7	Tamyanka 7	14.8	13.1	5.35	0.33	3.90
8	Mavrud 8	4.6	11.1	6.33	0.32	3.66
9	Mavrud 9	2.06	10.5	6.23	0.29	3.51
10	Tamyanka 10	87.0	7.6	11.95	3.15	3.66

The values of the titratable acids vary within a relatively wide range and correspond to the initial acids in the grapes. In all samples there is an increase in titratable acidity, which is explained by the predominance of synthesis over the metabolism of acids during fermentation. In the Tamyanka 10 sample, the increase in the titratable acidity is also related to the accumulation of high volatile acid concentrations. In the rest of the samples, the volatile acidity is within the normal range for spontaneously fermented wines.

The pH values demonstrate slight changes in relation to the initial grapes. The minor pH decrease in most samples is due to the increased acid formation.

In summary, the values of the main physicochemical parameters give reason to expect that the experimental grape samples have had a sufficient amount and stable yeast cells, which carried out vigorous and in most cases complete alcoholic fermentation, effectively converted sugars into alcohol, formed are normal amounts of volatile acidity.

In summary, the values of the main physicochemical parameters give reason to expect that sufficient amounts of resistant yeast cells have been present on the experimental grape

samples, which carried out vigorous and in most cases complete alcoholic fermentation, effectively converted sugars into alcohol, formed are normal amounts of volatile acidity. The Tamyanka 10 sample is an exception from most samples since the fermentation has beenhas been delayed, took a long time to start and could not finish; low alcohol has been formed; a lot of non-fermented sugars remained; the volatile acids formed have been high. The reasons for this behaviour of the sample could be found in the lack of sufficient and active wine yeast on the specific batch, the high sugar content and the osmotic shock related to that, as well as the possible presence of unfavourable or harmful microflora.

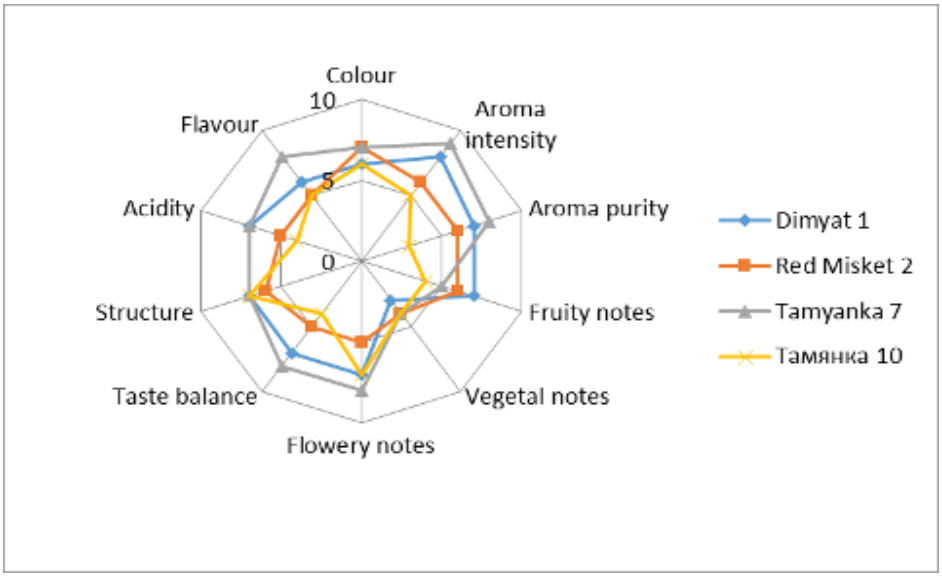


Figure 6. Sensory profile of white spontaneously fermented wines

The organoleptic analysis of the experimental wines made by a panel of 7 experienced oenologists gave sensory profiles of the variants presented in figures 6 and 7.

The colours of the white wines received low scores due to the existence of beige-brown nuances. This has been expected and attributed to the fermentation with solid parts and enrichment with phenolic compounds.

The intensity and purity of the aromas have been relatively well expressed; in the Dimyat 1 sample the fruit nuances predominated, whereas in the Tamyanka 7 sample the flowery notes have been the most pronounced. Flavour has been given the highest score in the Tamyanka 7 and Dimyat 1 samples: it has been intensive and fruity-flowery. On the whole, the taste in these samples has been balanced and the acids have been well integrated. The

Misket Cherven 2 sample has been slightly rough; the fermentation with the skins have had a stronger negative effect both on the smell and the taste. The problems with the alcohol fermentation progress in the Tamyanka 10 sample have had a negative effect on the sensory profile of the wine: it have had a lot of residual sugars, impure aroma and non-harmonic taste.

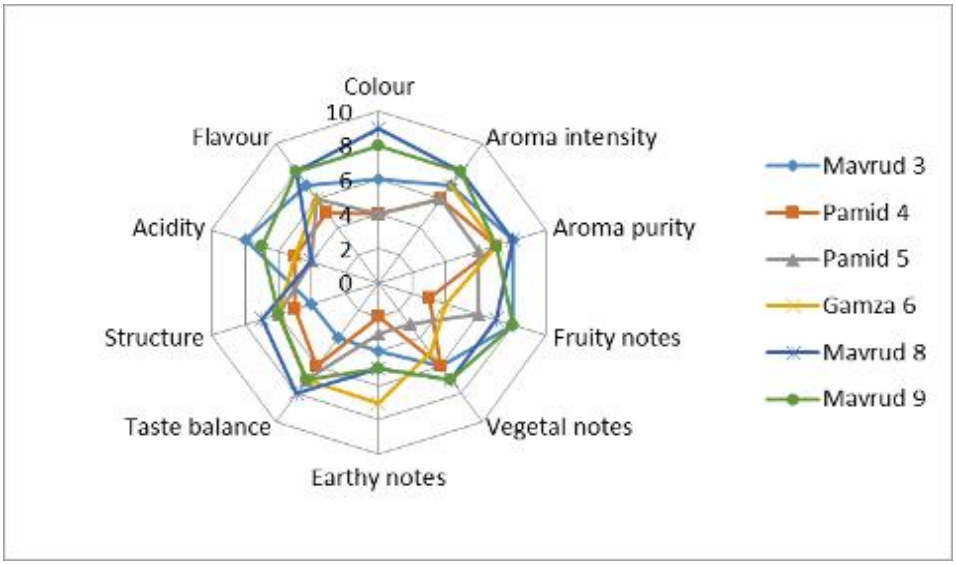


Figure 7. Sensory profile of red spontaneously fermented wines

The colour intensity of the red wines has been low in all samples, only higher in Mavrud 8. The aromas have been relatively pure, with average intensity. The aroma nuances in the Pamid wines have been one-dimensional, mainly vegetal; the earth notes dominated in Gamza 6, and fruity-vegetal nuances have been found in Mavrud 8 and Mavrud 9. The Mavrud 8 wine has been given the highest score in terms of taste, followed by Mavrud 9 and Gamza 6. All wines have had the sensory profile of dry wines, which has been a prerequisite for the existence of their own active wine yeast.

On the basis of the generalised results of the physicochemical and organoleptic analysis of the experimental spontaneously fermented wines, could be said that in all samples, with the exception of Tamyanka 10, active local wine yeast strains may be expected to be present. They have conducted dynamic, effective and complete alcohol fermentation; the metabolites formed have been within a normal range; the sensory profile has been positive for the experimental wines obtained under the specific conditions.

Isolating morphological types of spontaneously fermented experimental wines.

The growth on the complex medium has started as early as the 24-36th hour, whereas on the selective medium it has started at the 96th hour, in some of the samples only. Two of the variants, Misket Cherven 2 and Tamyanka 10, is presented in figures 8 and 9. The data on the growth of colonies on the individual media have been shown in Table 6.

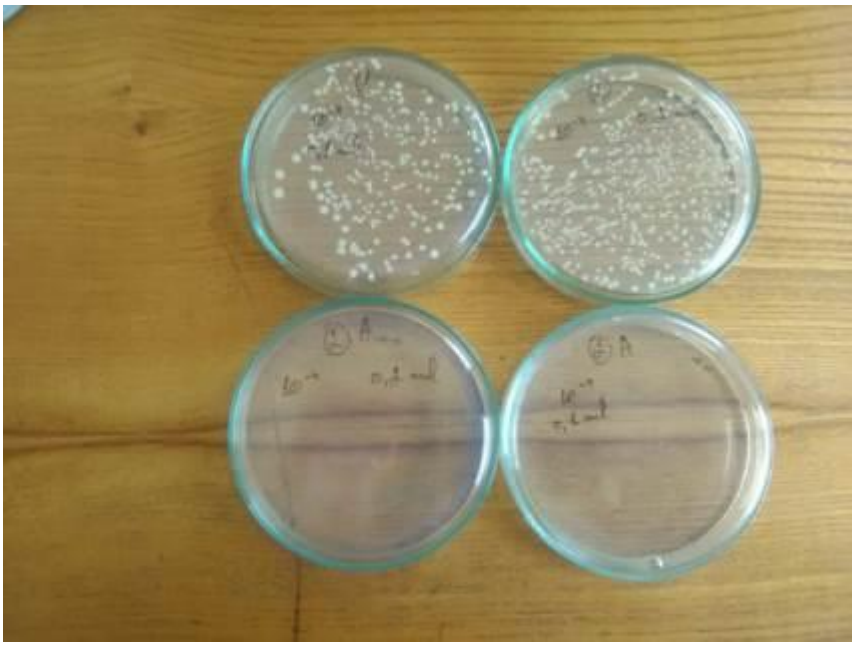


Figure 8. Inoculations from the Misket Cherven 2 sample.

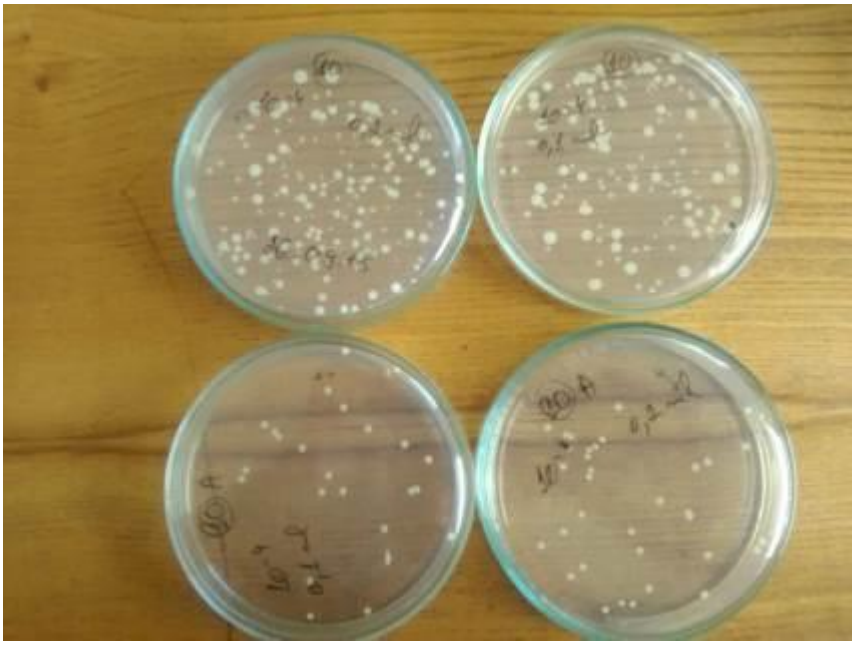


Figure 9. Inoculations from the Tamyanka 10 sample.

Table 6. Morphological types grown on complex and selective culture media

№	Source	Code	Colony description
1	Dimyat,village of Dimitrovche Svilengrad area	1-1 (D)	Large, smooth, semi-glistening, dome-shaped with a dimple, light cream-coloured, pasty, entire
2		1-2	Medium, smooth, glistening, dome-shaped with a dimple, light beige, pasty, with serrated edges
3		1-3-A	Small, cream-coloured, with serrated edges, semi-glistening, smooth, dome-shaped
4	Misket Cherven, village of Dimitrovche Svilengrad area	2-1 (D)	Large, smooth, semi-glistening, dome-shaped with a dimple, light cream-coloured, pasty, entire
5		2-2	Small, smooth, glistening, dome-shaped with a dimple, light cream-coloured, pasty, entire
6		2-3-A	Medium large, brownish, translucent towards the periphery, with a dimple, glistening, slimy
7		2-4-A	
8	Mavrud Lyubimets	3-1	Large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
9		3-2	
10		3-3-A	Medium large, brownish, translucent towards the periphery, with a dimple, glistening, slimy
11	Pamid Lyubimets	4-1 (D)	Large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
12		4-2	Small, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
13		4-3	Small, smooth, glistening, dome-shaped, pink, pasty, entire
14		4-4-A	Light cream-coloured, medium large, glistening, dome-shaped, slightly slimy
15	Pamid Malkata Zvezda village of Kolarovo	5-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
16		5-2	
17		5-3-A	Small, beige, glistening, pasty
18	Gamza Bratanovi Cellar village of Shishmanovo	6-1 (D)	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
19		6-2	Small, smooth, semi-glistening, dome-shaped, light beige, pasty, entire
20		6-3-A	Small, smooth, glistening, dome-shaped, white to light cream-coloured, pasty, entire
21		6-4-A	Large, smooth, glistening, dome-shaped, beige, slimy, entire
22	Tamyanka Bratanovi Cellar village of Shishmanovo	7-1 (D)	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
23		7-2	Small, smooth, semi-glistening, dome-shaped, light beige, pasty, entire
24		7-3-A (D)	Small, beige-brown, semi-glistening, sticky, smooth

25		7-4-A	Small, beige-brown, translucent, slightly slimy, smooth
26	Mavrud Bratanovi Cellar village of Shishmanovo	8-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
27		8-2	
28		8-3-A	Small, smooth, semi-glistening, dome-shaped, light beige, pasty, entire
29		8-4-A	
30	Mavrud Starosel Cellar village of Susam	9-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
31		9-2	
32		9-3	Small, cream-coloured to light beige, glistening, smooth, pasty
33		9-4	
34	Tamyanka Starosel Cellar village of Susam	10-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire
35		10-2 (D)	Small, beige, glistening, smooth, slightly slimy
36		10-3	Small, beige, glistening, smooth, slightly slimy, with a dimple
37		10-4-A (D)	Small, beige, strongly glistening, transparent in the periphery, smooth, dome-shaped
38		10-5	Medium, pink, glistening, smooth, dome-shaped

Note: (D) – dominant morphological type;

A – morphological type grown on an actidione containing medium.

A total of 38 morphological types have been isolated: 25 from complex media and 13 from selective media, respectively. In some cases, morphological types only differing in their colour hue or colony size have been isolated. This approach has been applied so that no present morphological type would be missed, and subsequent studies will aim to establish whether some of the isolated types are identical. The data in Table 4 show that there has been little variety of morphological types at the end of the process in all samples. This has been logical and could be expected. The microorganisms found have been probably markedly competitive and had replaced the others present on the grapes during the process. Perhaps those have been the microorganisms which had performed the main part of the alcoholic fermentation and determined, through their metabolism, the physicochemical composition and sensory profiles of the experimental wines obtained. More morphological types have been established in the Tamyanka 10 sample; probably there have been no strong antagonists there, which enabled the participation of different types of microorganisms throughout the process.

Morphological and cultural characterisation of newly isolated morphological types from spontaneously fermented experimental wines.

The morphological characterisation has been made on 72-hour liquid cultures, and the cultural characterisation on 72-96-hour colonies formed on grape juice agar. The results are presented in Table 7.The cell sizes of the isolated yeast strains have been similar, varying from 3-6µm in width to 4-10µm in length.

Table 7. Morphological and cultural characteristics of the isolated strains

№	Source	Code	Colony description	Microorganism type yeast/bacterium	Cell morphology
1	Dimyat, village of Dimitrovche Svilengrad area	1-1	Large, smooth, semi-glistening, dome-shaped with a dimple, light cream-coloured, pasty, entire	Y	Oval-ellipsoid cells, individually located in the medium; vegetative propagation by budding: polar, unilateral;
2		1-2		Y	
3		1-3-A	Small to medium, beige to light brown, with serrated edges, glistening, smooth, transparent towards the periphery, dome-shaped.	B	Ellipsoid shape of the cells, located in twos in the medium, tremulous;
4	Misket Cherven, village of Dimitrovche Svilengrad area	2-1	Large, smooth, semi-glistening, dome-shaped with a dimple, light cream-coloured, pasty, entire	Y	Spherical-oval cells, individually located in the medium; vegetative propagation by budding: polar, unilateral;
5		2-2	Small to medium, smooth, semi-glistening, dome-shaped, light beige, pasty, entire	Y	
6		2-3-A	Small, to medium large, brownish, translucent towards the periphery, with a dimple, glistening, slimy	B	Ellipsoid to rod-shaped shape of the cells, located in twos in the medium, tremulous;
7		2-4-A		B	
8	Mavrud Lyubimets	3-1	Large, smooth, semi-glistening, dome-shaped, cream-coloured to light beige, pasty, entire, with a brownish dimple in the centre	Y	Oval-ellipsoid cells, individually located in the medium; vegetative propagation by budding: polar, unilateral;
9		3-2		Y	

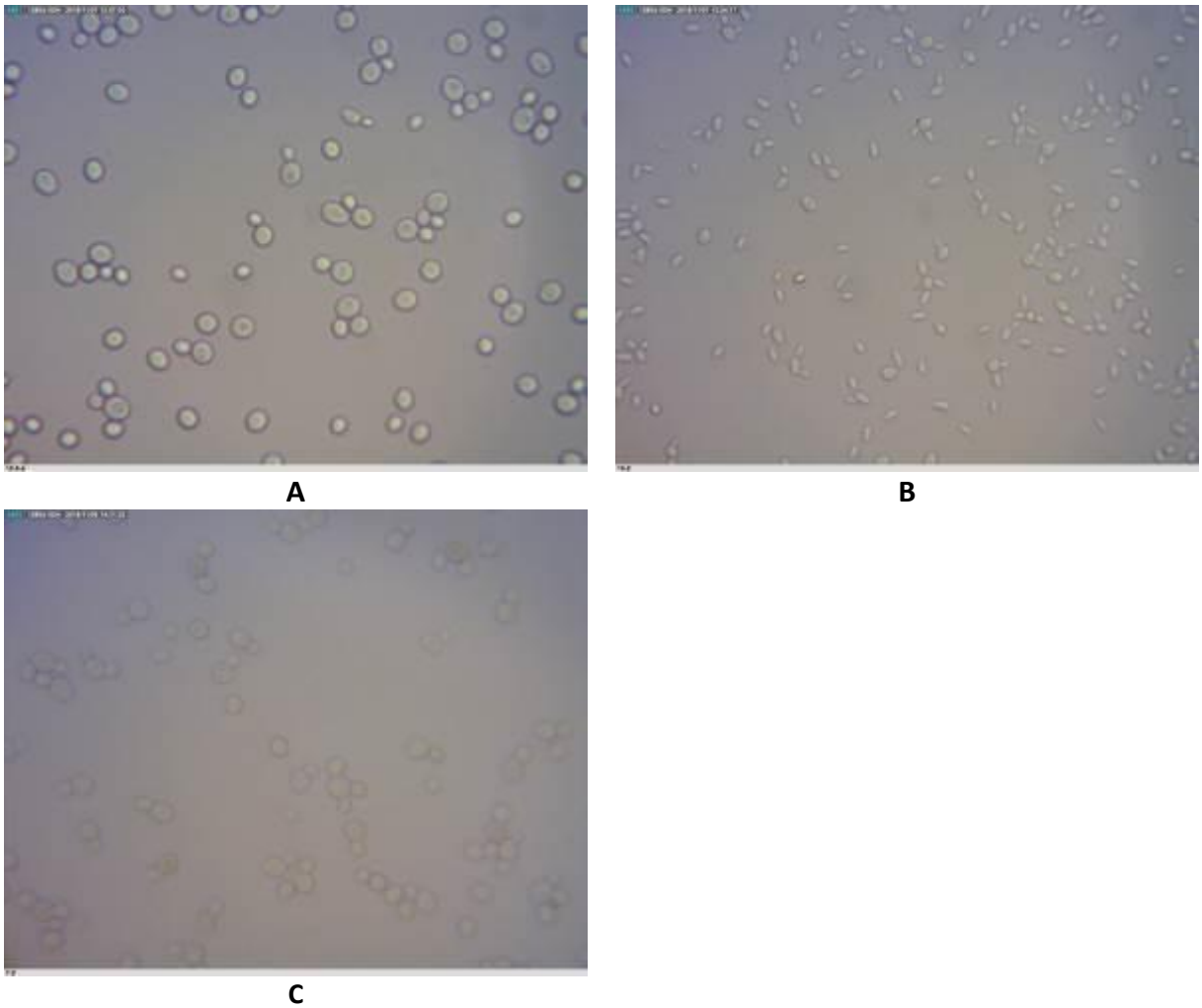
10		3-3-A	Medium large, brownish, translucent towards the periphery, with a dimple, smooth, strongly glistening, slimy	B	Ellipsoid shape of the cells, located in twos in the medium, tremulous;
11	Pamid Lyubimets	4-1	Large, smooth, semi-glistening, dome-shaped with a dimple in the centre, cream-coloured, pasty, entire	Y	Oval-spherical to ellipsoid cells, individually located in the medium; vegetative propagation by budding: polar, unilateral;
12		4-2	Small, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	
13		4-3		Y	Spherical-oval cells, individually located in the medium, a little smaller than 4-1 and 4-2; vegetative propagation by budding: polar, unilateral;
14		4-4-A	Light beige, medium large, glistening, dome-shaped, slightly slimy, translucent	B	Ellipsoid shape of the cells, located in twos in the medium, tremulous;
15	Pamid Malkata Zvezda village of Kolarovo	5-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Oval-ellipsoid cells, individually located in the medium; vegetative propagation by budding: unilateral, polar and slightly to the side;
16		5-2		Y	
17		5-3-A	Small, beige, dome-shaped, glistening, slightly slimy	B	Coccoid to ellipsoid shape of the cells, located mainly in fours in the medium, immobile;
18	Gamza Bratanovi Cellar village of Shishmanovo	6-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Oval-ellipsoid cells, individually located in the medium; vegetative propagation by budding: unilateral, polar;
19		6-2		Y	
20		6-3-A	Small, smooth, glistening, dome-shaped, white to light cream-coloured, pasty, entire	Y	Spherical to oval cells, a little smaller than 6-1 and 6-2, individually located in the medium; vegetative propagation by budding: unilateral, polar, more rarely multilateral;
21		6-4-A	Small, smooth, glistening, dome-shaped, pearlescent, slimy, entire.	B	Coccoid shape of the cells, located in twos and in chains in the medium, immobile;

22	Tamyanka Bratanovi Cellar village of Shishmanovo	7-1	Medium to large, smooth, semi-glistening, dome-shaped, light beige, pasty, entire	Y	Ellipsoid-oval cells, individually located in the medium; vegetative propagation by budding: multilateral;
23		7-2		Y	
24		7-3-A	Small, beige-brown, semi-glistening, sticky, smooth	B	Coccoid to ellipsoid shape of the cells, a little larger than 4-4-A, located in twos in the medium, more rarely in tetrads, immobile;
25		7-4-A	Small, beige-brown, translucent, slightly slimy, smooth, with a dimple	B	Ellipsoid shape of the cells, smaller than 7-3-A, located in twos in the medium, tremulous;
26	Mavrud Bratanovi Cellar village of Shishmanovo	8-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Oval до ellipsoid cells, individually located in the medium; vegetative propagation by budding: unilateral, polar;
27		8-2		Y	
28		8-3-A	Small, smooth, semi-glistening, dome-shaped, beige, entire	B	Ellipsoid shape of the cells, located in twos in the medium, tremulous;
29		8-4-A	Very small, smooth, pearlescent, transparent, glistening	B	Coccoid shape of the cells, located in twos and in chains in the medium, immobile;
30	Mavrud Starosel Cellar village of Susam	9-1	Large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Spherical-oval to ellipsoid cells, individually located in the medium; vegetative propagation by budding: unilateral, polar and a little to the side;
31		9-2		Y	
32		9-3		Y	
33		9-4		Y	
34	Tamyanka Starosel Cellar village of Susam	10-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Ellipsoid-oval cells, individually located in the medium; vegetative propagation by budding: polar, unilateral;
35		10-2	Small to medium, beige, glistening, smooth, slightly slimy	Y	Ellipsoid, slightly elongated cells, individually located in the medium, smaller than 10-1; vegetative propagation by budding: unilateral, polar and a little to the side;
36		10-3	Small, cream-coloured to light beige, semi-glistening, smooth, with a dimple, entire	Y	Oval-spherical cells, larger than 10-1, individually located in the medium; vegetative propagation by budding: polar, unilateral;

37		10-4-A	Small, beige to light brown, strongly glistening, transparent in the periphery, smooth, dome-shaped	B	Coccoid to ellipsoid shape of the cells, located in twos in the medium, tremulous;
38		10-5	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured to light beige, pasty, entire.	Y	Like 10-3: oval-spherical cells, larger than 10-1, individually located in the medium; vegetative propagation by budding: polar, unilateral;

Note: **Y:** yeast, **B:** bacteria

Microscopic images of some of the yeast strains studied have been shown in figure 10.



A **B**

C

Figure 10. Native microscopic preparation of part of the isolated yeast strains: 10-5-A (A), 10-2 (B) and 7-2 (C).

In most cases, the yeast cells are spherical to slightly ellipsoid, located individually in the medium; they reproduce vegetatively by budding, most often unilateral, polar (picture A). In some of the strains the cells are quite smaller, elongated, and slightly sharpened (picture B), and multilateral budding could be observed at places (picture C). Frequently during the morphological and cultural characterisation, pronounced similarity or identity has been established between morphological types isolated as different. The inoculum density have a strong effect on the colony size, and the culture age affects the colour.

The yeast growth on a liquid culture medium, grape juice, has been similar. The propagation starts around the 18-24th hour; the medium has been slightly turbid; individual gas bubbles have been released; at the 36th hour the medium has been very turbid, and foam, most often fine-grained, started to form in different amounts. Around the 72nd hour, sediment started to form. Its quantity increased until the 168th hour; it thickened and became fatty. The flocculation of the individual strains varied slightly, without any particular trend, and the liquid in the test tubes has been clarified for different periods and to varying degrees with the different strains.

The growth on a solid culture medium has been very close with the different strains. The sizes of their colonies and their colour hues varied slightly.

During the microscopic characterisation of the bacteria, most often cocci, slight ellipses and more elongated ellipses have been most frequently observed. Propagation has been by division everywhere; in some cultures the cells have been located in twos (10-4-A; 7-4-A), elsewhere short chains of ellipsoid cocci (8-4-A; 6-4-A) and sometimes tetrads (5-3-A; 7-3-A) have been observed.

The growth of the bacteria on a liquid culture medium has been close and very similar: poor growth, almost no turbidity of the medium, no significant sediment has been formed, slight clouds have been visible when the test tubes have been stirred, and in some cases a light membrane has been formed on the liquid surface (8-3-A; 10-4-A).

The colonies of the bacteria have been very small, under 0.1 mm in diameter, transparent, slightly slimy, strongly glistening, some of them pearlescent (6-4-A; 8-4-A), and others light beige (8-3-A; 10-4-A).

Spore formation in newly isolated yeast strains from spontaneously fermented experimental wines.

The spore formation in newly isolated yeast strains has been studied according to a classical method: development of a single colony on a complex solid culture medium, biomass transfer onto agar without a carbon source, storage at 25°C for 6 days, and microscopic examination of a biomass sample.

The results of the examination have been presented in Table 8.

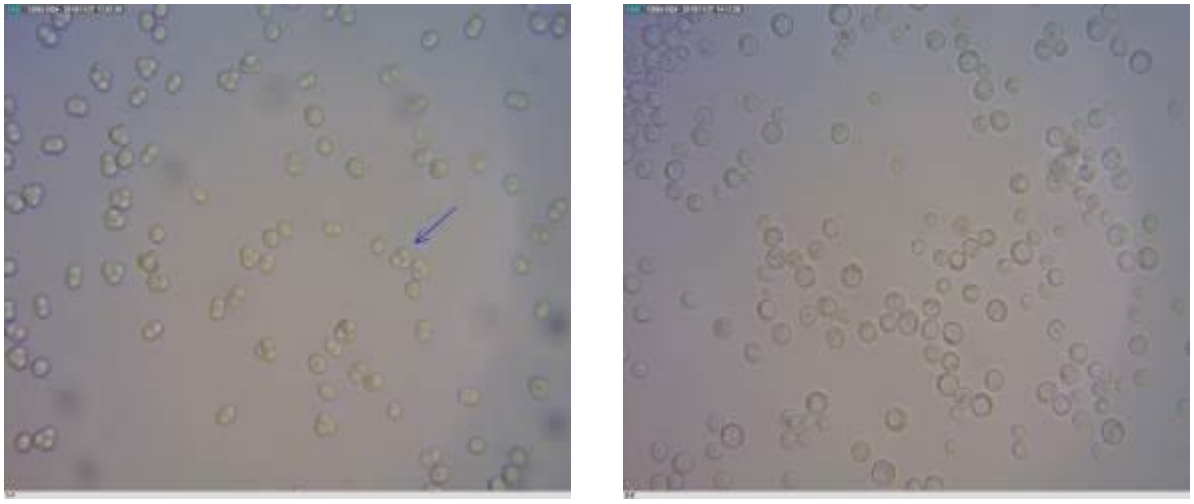
Table 8.Spore formation in newly isolated yeast strains

№	Source	Code	Microscope results, spore formation
1	Dimyat, village of Dimitrovche Svilengrad Region	1-1	Abundant spore formation, mainly 3 spores per ascus, *
2		1-2	Abundant spore formation, mainly 3 spores per ascus, *
3	Misket Cherven, village of Dimitrovche Svilengrad Region	2-1	Abundant spore formation, mainly 3, rarely 4 spores per ascus, *
4		2-2	Very poor or did not form spores on agar without a carbon source
5	Mavrud Lyubimets	3-1	Rarely forms spores, 1–2spores per ascus, *
6		3-2	Very rarely forms spores, 1–2 spores per ascus, *
7	Pamid Lyubimets	4-1	Very rarely forms spores, 1–2 spores per ascus, *
8		4-2	Fairly abundant spore formation, 1–2 spores per ascus,*
9		4-3	Abundant spore formation, 3–2 spores per ascus, *
10	Pamid Malkata Zvezda village of Kolarovo	5-1	Poor to average spore formation, 2–4 spores per ascus, *
11		5-2	Poor to average spore formation, 2–4 spores per ascus, *
12	Gamza Bratanovi Cellar village of Shishmanovo	6-1	Abundant spore formation, 2–4 spores per ascus, *
13		6-2	Abundant spore formation, 2–4 spores per ascus, *
14		6-3-A	Abundant spore formation, from 1 to 4 spores per ascus, *
15	Tamyanka Bratanovi	7-1	Abundant spore formation, 2–4 spores per ascus, *
16		7-2	Abundant spore formation, 2–4 spores per ascus, *

17	Cellar village of Shishmanovo	7-3	Abundant spore formation, most often 3 spores per ascus,*
18	Mavrud Bratanovi Cellar village of Shishmanovo	8-1	Fairly abundant spore formation, 2 – 3 spores per ascus, *
19		8-2	Abundant spore formation, from 2 – 4 spores per ascus, *
20	Mavrud Starosel Cellarvillage of Susam	9-1	Fairly abundant spore formation, 2– 3 spores per ascus, *
21		9-2	Abundant spore formation, 2 – 3 spores per ascus, *
22		9-3	Fairly abundant spore formation, from 2–4 spores per ascus, *
23	Tamyanka Starosel Cellar village of Susam	10-1	Fairly abundant spore formation, from 2–3 spores per ascus, *
24		10-2	Cells very rarely form spores, 1–2 spores per ascus, *
25		10-3	Abundant spore formation, from 2 – 4 spores per ascus, *
26		10-5	Medium-abundant spore formation, 2– 3 spores per ascus, *

* - spore formation without previous copulation

Almost all newly isolated yeast strains formed spores, most frequently fairly abundant to abundant: between 50-60% and 70-80 % of the cells form spores. No spore formation has been observed with strain 2-2 only, whereas strain 10-2 exhibited very poor spore formation. Everywhere, spore formation ran without previous copulation, and 2 – 4 spores have been most often observed in the asci. The spores are spherical to oval, and in some cases they slightly deform the ascus.Figure 11 presents microscopic images of some of the strains studied.



A **B**

Figure 11. Microscopic image of spore formation: 1-1 (A), 2-2 (B).

Fermentation of sugars by newly isolated yeast strains from spontaneously fermented experimental wines.

As an element of the characterisation of the newly isolated yeast strains and, more specifically, their identification according to classical methods, their ability to ferment sugar has been also studied. The chromatographic method widely applied in microbiological studies (Ivanov, Trifon et al. A Practicum in Wine Technology, Plovdiv: Hristo G. Danov Publishers, 1979) has been used. Fermentation ability has beenexamined with regard to glucose, fructose, galactose, sucrose, maltose and raffinose in a 0.6% yeast extract solution. The chromatograms of some of the strains studied have been shown in figure 12.

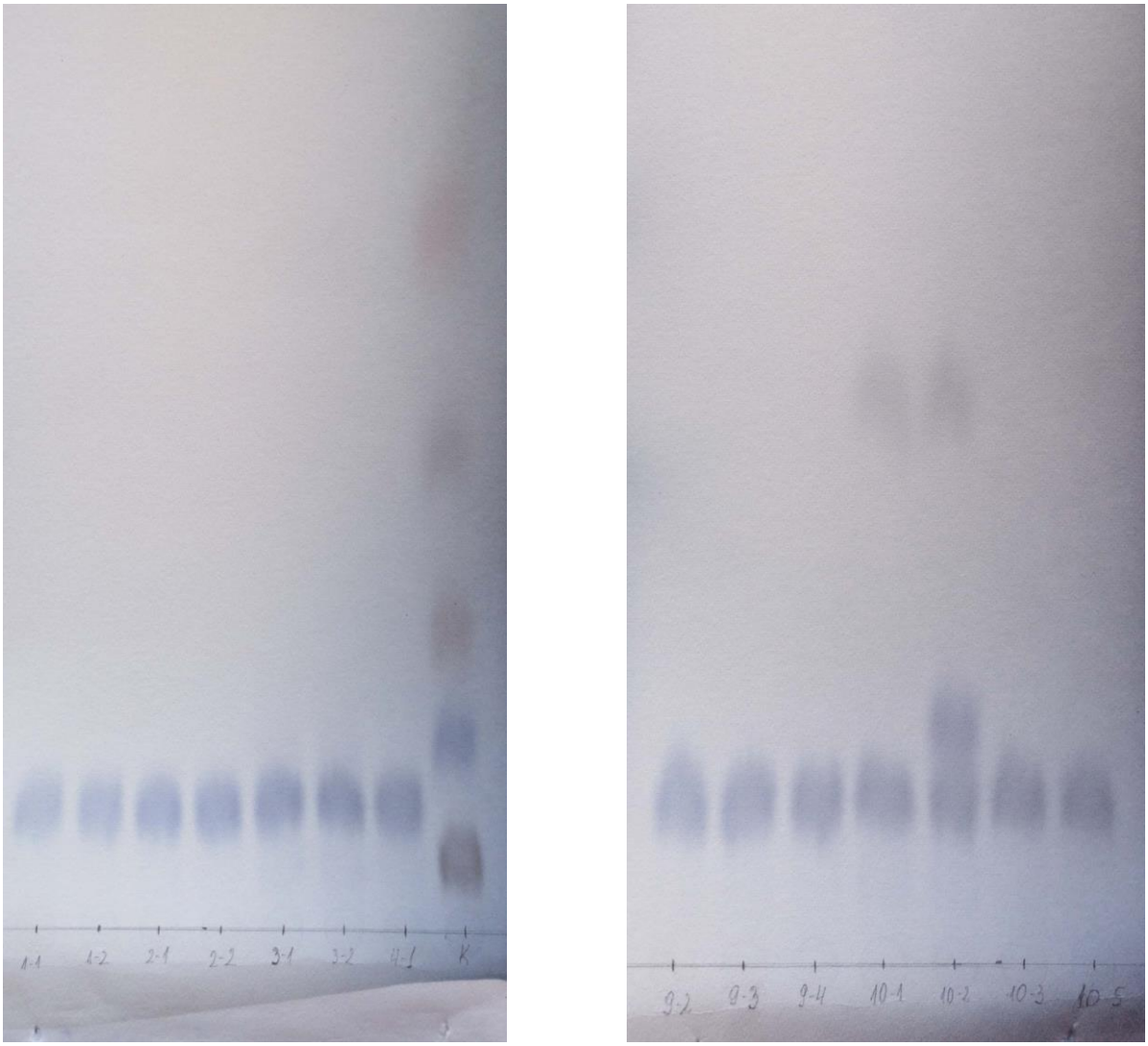


Figure 12. Chromatograms for sugar fermentation.

The main part of the yeast strains ferments glucose, fructose, galactose, sucrose, maltose, and 1/3 raffinose. These are strains 1-1, 1-2, 2-1, 2-2, 3-1, 3-2, 4-1, 4-2, 4-3, 5-1, 5-2, 6-1, 6-2, 6-3A, 7-1, 7-2, 8-1, 8-2, 9-1, 9-2, 9-3, 9-4, 10-3 and 10-5. Out of the above sugars, strains 10-1 and 10-2 does not ferment galactose, and 7-1, 7-2, and especially 10-2, take longer to ferment maltose.

On the basis of the morphological, cultural and physiological characteristics esatablished and Kurtzman'staxonomy (2011), we could attribute the strains studied to the genera and species specified in Table 9 with a high degree of probability.

Table 9. Determination of newly isolated yeast strains by genera and species

№	STRAIN	GENUS, SPECIES
1	1-1, 1-2, 2-1, 2-2, 3-1, 3-2, 4-1, 4-2, 4-3, 5-1, 5-2, 6-1, 6-2, 6-3A, 7-1, 7-2, 8-1, 8-2, 9-1, 9-2, 9-3, 9-4, 10-3, 10-5	Saccharomyces cerevisiae(ellipsoideus)
2	10-1, 10-2	Saccharomyces cerevisiae(oviformis)

As can be seen from the data in Table 9, all newly isolated strains can be attributed to the typical wine yeast *Saccharomycescerevisiae* according to Kurtzman (2011), 92% of them belonging to *Saccharomycescerevisiae* (*ellipsoideus*) according to Lodder (1970), and only 8% belonging to *Saccharomycescerevisiae* (*oviformis*) according to Lodder. This hypothesis is in conformity with the data in literature that the yeasts of the first species are the ones that are much more widely distributed in nature.

2. PCR analysis of newly isolated yeast strains.

Part of the newly isolated yeast strains have been subjected to PCR analysis. Representatives of all isolation sources and the maximally possible number of grape varieties have been selected. The primers Δ-12 and Δ-21, which are very specific for the *Saccharomycescerevisiae* species, have been used. The PCR product obtained has been separated via gel-electrophoresis and visualised after UV radiation (figure 13).

Eight different profiles can be practically distinguished in the figure:

- First one with 4 segments, 170, 220, 320 and 530 base pairs in size, respectively;

- Second one with 5 segments, 170, 220, 300, 320 and 530 base pairs in size, respectively;
- Third one with 4 segments, 170, 250, 300 and 530 base pairs in size, respectively;
- Fourth one with 3 segments, 170, 220 and 530 base pairs in size, respectively;
- Fifth one with 3 segments, 170, 460 and 530 base pairs in size, respectively;
- Sixth one with 3 segments, 170, 400 and 530 base pairs in size, respectively;
- Seventh one with 6 segments, 120, 180, 700, 850, 950 and 1250 base pairs in size, respectively;
- Eighth one with 6 segments, 120, 400, 650, 850, 1200 and1250 base pairs in size, respectively.

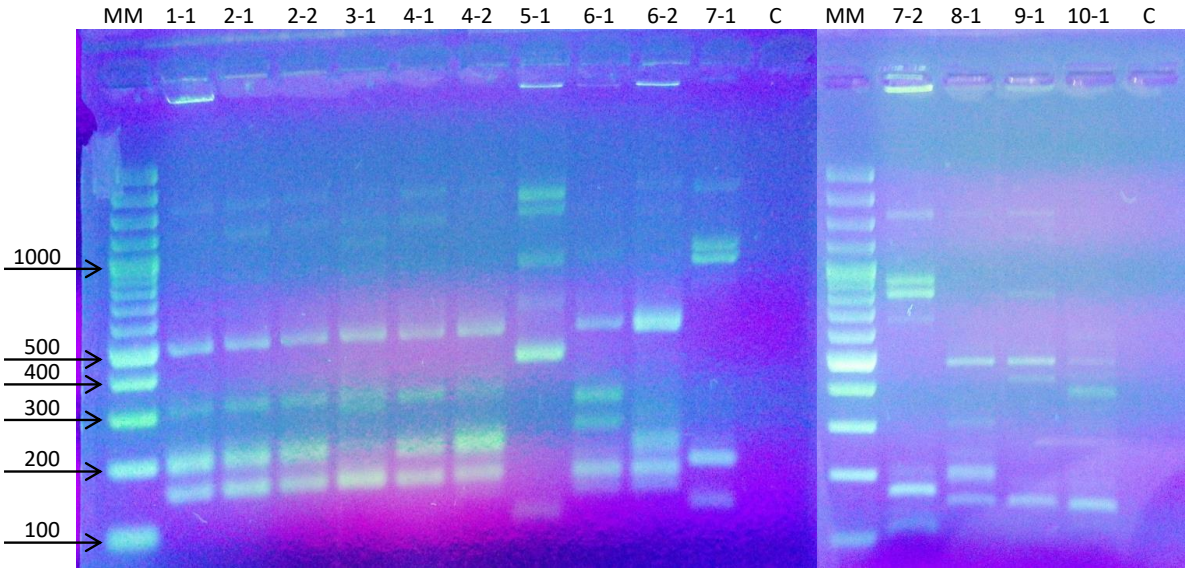


Figure 13. Visualisation of a PCR product from newly isolated yeast strains

The progress of the PCR reaction and the obtaining of a PCR product, profiles respectively, made it possible to attribute the newly isolated yeast strains to the *Saccharomycescerevisiae* species. Those results have been in conformity with the results obtained through classical identification.

The comparative analysis of the profiles established and the location of the isolation sources provided interesting grounds for discussion.

The first profile comprises strains 1-1, 2-1, 2-2, 4-1, 4-2 and 8-1. They have been from three sources: 1-1, 2-1, 2-2 have been from the region of thevillage of Dimitrovche, 4-1 and 4-2 have been from Lyubimets, and 8-1 has been from the village of Shishmanovo. On this basis, it could be stated that yeasts with very close profiles have been distributed in these relatively closely situated regions.

The second profile included strain 3-1 (source: Lyubimets), the third 6-1 (source: village of Shishmanovo), the fourth 6-2 (source: village of Shishmanovo), the fifth 9-1 (source: village of Susam), and the sixth profile included strain 10-1 (source: village of Susam). It is evident that even within one country, there are yeasts with different profiles, and that even within a small micro-region there are yeasts with different profiles. The last statement has been confirmed by the seventh profile type established in strains 7-1 and 7-2, also isolated from the region of the village of Shishmanovo. This profile differed significantly from the third and fourth profiles from the same micro-region.

The eighth profile has been established for strain 5-1 isolated from a more distant region, the village of Kolarovo. It differed greatly from all other profiles.

It also has to be pointed out that the yeast strains studied have not been accidentally present on the grapes; they have been stable, fermentationally active, powerful antagonists; they have replaced the other yeast on the grape batches; they have conducted the spontaneous alcoholic fermentation to the end.

On the one hand, the similarity in profiles enables us to believe that these strains are very similar to one another and we could expect identical behaviour from these yeasts. We could even suggest that these yeasts are branches of the same strain. On the other hand, we cannot claim with certainty that they are identical or expect the same behaviour from them. The reason for that is that figure 13 visualises the results obtained from the analysis of only one part of the yeast cell DNA, not the whole DNA.

It can also be seen that the majority of yeasts having similar profiles had been isolated from the same or close micro-regions. This shows that one yeast type dominates a given vineyard. In more geographically remote vineyards we find different profiles, which however exhibit

some similarities. This shows that branches of certain yeast strains can even be found at large distances from one another. The distribution of individual yeast strains is strongly affected by natural and climatic conditions: air currents, rainfall, birds, insects, and other factors. Their competitiveness under real natural conditions, their ability to replace other strains in the fight for a substrate, to propagate and predominate as indigenous microflora are also of great significance.

Characteristics of newly isolated bacterial strains

In the process of isolation of microorganisms from spontaneously fermented wines from the grape batches studied, a total of 12 bacterial strains have been isolated as presented in Table 10.

Table 10. Newly isolated bacterial strains

Sample	Variety	Town/Village	Bacterial strain
1	Dimyat	village of Dimitrovche, Svilengrad Municipality	1-3-A
2	Misket Cherven	village of Dimitrovche, Svilengrad Municipality	2-3-A; 2-4-A
3	Mavrud	Lyubimets	3-3-A
4	Pamid	Lyubimets	4-4-A
5	Pamid	village of Kolarovo, Malkata Zvezda Cellar	5-3-A
6	Gamza	village of Shishmanovo, Bratanovi Cellar	6-4-A
7	Tamyanka	village of Shishmanovo, Bratanovi Cellar	7-3-A; 7-4-A
8	Mavrud	village of Shishmanovo, Bratanovi Cellar	8-3-A; 8-4-A
9	Mavrud	village of Susam, Starosel Cellar	-
10	Tamyanka	village of Susam, Starosel Cellar	10-4-A

All bacterial cultures have been isolated from a selective culture medium containing actidione. The cultures have been inoculated in a sterile bacterial medium, stored in a refrigerator and studied for the following: Gram staining, catalase activity, acetic acid formation from ethanol, acid formation from glucose, veil formation on an ethanol-containing medium. The results of the study have been summarised in Table 11.

Table11.Characteristics of newly isolated bacterial strains

№	Strain	Gram staining	Catalase activity	Acetic acid formation from ethanol	Veil formation on an ethanol-containing medium
1	1-3-A	„–“	yes	yes	yes
2	2-3-A	„–“	yes	yes	yes
3	2-4-A	„–“	yes	yes	yes
4	3-3-A	„–“	yes	yes	yes
5	4-4-A	„–“	yes	yes	yes
6	5-3-A	„+“	no	no	no
7	6-4-A	„+“	no	no	no
8	7-3-A	„+“	no	no	no
9	7-4-A	„–“	yes	yes	yes
10	8-3-A	„–“	yes	yes	yes
11	8-4-A	„+“	no	no	no
12	10-4-A	„–“	yes	yes	yes

Table 12. Typeand genus determinationof newly isolated bacterial strains

№	Strain	Type	Genus
1	1-3-A	Acetic acid bacteria	Acetobacter
2	2-3-A	Acetic acid bacteria	Acetobacter
3	2-4-A	Acetic acid bacteria	Acetobacter
4	3-3-A	Acetic acid bacteria	Acetobacter
5	4-4-A	Acetic acid bacteria	Acetobacter
6	5-3-A	Lactic acid bacteria	Pediococcus
7	6-4-A	Lactic acid bacteria	Leuconostoc
8	7-3-A	Lactic acid bacteria	Oenococcus
9	7-4-A	Acetic acid bacteria	Acetobacter
10	8-3-A	Acetic acid bacteria	Acetobacter
11	8-4-A	Lactic acid bacteria	Leuconostoc
12	10-4-A	Acetic acid bacteria	Acetobacter

On the basis of the results from the study of the morphological, cultural and some biochemical characteristics of the newly isolated bacterial strains, a motivated hypothesis

may be made regarding their genus and, to some extent, their species. The information has been presented in Table 12.

It is evident that 2/3 of the bacterial strains could be classified as acetic acid bacteria, and 1/3 as lactic acid bacteria. All strains in the acetic acid group have been attributed to the Acetobactergenus, whereas there has been much greater variety in the lactic acid group. With regard to the stronger antagonism of yeast in relation to bacteria and the moment of isolation, i.e. the end of the alcoholic fermentation, it could be expected that the bacterial strains identified would be fewer and relatively uniform.

CONCLUSION: As a result of a contract signed underthe Developing Identity ON Yield, SOil and Site project with the Institute of Viticulture and Oenology in Pleven (IVO Pleven) and the task assigned from Work Package 3 (WP3)**/ Defining the wine's own identity in the region studied: Advantage of the region,** Activity3.2 **Selection of main local wine varieties and determination of their characteristics**,10 grape batchesfrom different regions have been studied for present microflora (yeast, bacteria). Grape mash from 10 grape batches have been subjected to spontaneous fermentation, and analyses have been made of the physicochemical characteristics of the resultant wines, along with sensory characterization. Thirty-eight morphological types, including 26 yeast strains and 12 bacterial strains, have been isolated and their morphological and cultural characteristics have been determined. The yeast strains have been checked for spore formation and sugar fermentation, and the strain variety of part of them has been determined using the molecular PCR method. The bacterial strains have been subjected to a number of studies for the purpose of their type and genus identification.

A general conclusion can be made that yeast cultures differing in characteristics and properties have been established, all of them having wine yeast specificity and high fermentation capacity. Strains similar or different in their DNA structure have been established in the individual micro regions. It can be expected that specific regional wines having suitable composition and sensory profiles can be produced using the local yeast microflora.

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%BF%D1%80%D0%BE%D0%B1%D0%B8%20%D0%B7%D0%B0%20%D0%B8%D0%B4%D0%B5%D0%BD%D1%82%D0%B8%D1%84%D0%B8%D0%BA%D0%B0%D1%86%D0%B8%D1%8F_22062020_%D0%9F%D0%A0%D0%97%D0%A2%D0%9A.pdf.

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