Morphological and cultural characteristics and sporulation in microorganisms isolated from spontaneously fermented wines

Tatyana Yoncheva[1*], *Iliyan* Simeonov[1], *Nikolay* Genov[1], *Vladimir* Dimitrov[1], *Dimitar* Dimitrov[1], *Hristo* Spasov[2], *Nadezhda* Blagoeva[2] and *Polina* Zapryanova[2]

[1]Agricultural academy, Institute of Viticulture and Enology – Pleven, Bulgaria
[2]University of Food Technologies – Plovdiv, Bulgaria

Abstract. The morphological and cultural characteristics of newly isolated yeast and bacterial strains and the process of sporulation in yeasts, were studied. As a result of spontaneous alcoholic fermentation of grape pomace from 10 samples of grapes from autochthonous white and red vine varieties, a total of 38 morphological types were isolated. A morphological and cultural characterization of the isolated pure cultures of microorganisms was made, of which 26 yeast and 12 bacterial strains. For the investigation, 72-hour liquid cultures grown in sterile grape juice medium and colonies formed on grape juice–agar medium were used. In shape, the yeast cells were spherical to slightly elliptical, located singly in the medium. They multiplied by budding, most often unilateral, polar. Cell sizes ranged from 3-6 µm to 4-10 µm in length. The growth of the different strains of yeast and bacteria in liquid and on solid nutrient media was similar. Their propagation started around 18-24 hours. The flocculation of individual yeast strains varied, with no definite trend. A sporulation process was observed in yeast. Almost all isolated strains produced spores without prior copulation. Sporulation was most often quite abundant to abundant, about 50-60% and 70-80% of the cells formed spores.

1 Introduction

The microbial ecosystem of grapes and wine, including yeasts of the *Saccharomyces* and non-*Saccharomyces* species, as well as lactic acid bacteria, is considered a crucial factor for winemakers and oenologists, influencing wine aroma and consumer preferences [1, 2, 3]. The number of microbial populations on the grapes depends on the variety, the degree of ripeness, the harvest and the conditions of the year. Therefore, grape microbiota could vary not only between different varieties but also between each plant and grape cluster [2, 4, 5, 6].

Of the grape microflora, involved in the fermentation kinetics and influencing the wine composition and characteristics, the most important are yeast and bacteria. Bacteria are unicellular prokaryotic organisms that reproduce by cell division and rarely by budding. Yeasts are unicellular eukaryotes that reproduce primarily vegetatively by budding and by sporulation [7, 8]. Yeasts are particularly significant in winemaking because they carry out the main process of alcoholic fermentation. Their autolytic products might affect the chemical composition and sensory qualities of the wine and influence the growth of malolactic and other bacteria. Prominent in the winemaking process are *Saccharomyces* species (predominantly *Saccharomyces cerevisiae*) which dominate the alcoholic fermentation [6, 9, 10].

*Corresponding author: t_yoncheva@ive.bg

Selected yeasts have certain technological characteristics that make them suitable for industrial wine production. In the last few years there has been an increasing interest in local selected yeast for controlled must fermentation. Autochthonous strains are presumable to be more competitive because they are better acclimated to the environmental conditions. Selection of the appropriate local yeast along with the terroir features of the area assure the maintenance of the typical composition and sensory properties of the wines produced in each region [11, 12, 13, 14, 15].

The study of microorganisms and their identification cover their morphological, cultural and physiological properties. The main morphological characteristics include shape and size of the cell, mode of vegetative propagation, structure, sporulation and coloration of the spores, etc. Yeasts are characterized by a variety of cell shapes - globular, oval, ellipse-shaped, lemon-shaped located mostly singly or in groups. Bacteria are spherical (cocci) and rod-shaped. Monococci, diplococci, tetrads and streptococci are found in wine. The rod-shaped bacteria have the shape of short or long rods, most often arranged in chains [4, 5, 16, 17.].

Microorganisms overcome unfavourable conditions for their existence by sporulation. When yeast cells grow in specific low nutrient content, nitrogen starvation and the presence of a poor carbon source, sporulation may be initiated and meiotic division produces haploid stress resistant spores contained

inside a protective ascus. The spores do not acquire new resources but are partially protective against critical factors. One ascus might contain 1-8 spores of globular, bean-shaped, hat-shaped and hemispherical shapes [16, 18, 19]. Spore formation is not observed in all bacteria species. Only cells that develop under optimal conditions sporulate with one spore [20].

The cultural properties of microorganisms include their growth in liquid and on solid nutrient media [17, 21]. For yeast in a liquid medium (grape juice), it is monitored whether and when fermentation begin – intensity of gas releasing, foam formation, sedimentation and nature of the sediment. Colonies of various shapes and sizes develop on a solid nutrient medium (grape juice agar). Their structure and appearance are studied. The colouring of the colonies is versatile – from white, cream, yellow, pink to various shades of green. For bacteria in a liquid medium (grape juice/wine), it is studied their ability to form a pellicle, sediment, to turbid the medium, giving it a smell. On a solid medium, the formation of colonies, their sizes, colour, shape, surface, profile, periphery and structure are studied [9, 11, 13].

The identification of individual yeast species could be achieved not only by morphological tests but also with some physiological ones, which include the measurement of assimilation of individual carbon and nitrogen sources by auxanography, fermentation test, ethanol tolerance, osmotolerance or thermotolerance [21].

Cultural conditions have a great influence on yeast growth. The effect of stress is frequently to suppress growth rate and product formation. The pH value could change the ionization degree of media nutrients, affecting cellular nutrient absorption and ultimately affecting growth. Also high sugar concentration could inhibit yeast growth. The high osmotic pressure and increased sugar content lead to water loss from yeast cells and decrease their activity [17, 22, 23].

The isolation and identification of autochthonous yeast strains from locally fermented wines and the characterization of their taxonomic and phenotypic diversity is very important for determining the oenological qualities of wine [12, 24, 25, 26, 27]. The objective of the study was to investigate the morphological characteristics, cultural properties and sporulation in microorganisms isolated from spontaneously fermented wines from different micro-regions.

2 Material and Methods

2.1 Grapes processing and alcoholic fermentation

2.1.1 Origin and composition of grapes from the experimental samples

Grapes from 3 white (Dimyat, Red Misket, Tamyanka) and 3 red (Pamid, Gamza, Mavrud) autochthonous wine varieties from 5 different micro-regions of Haskovo Region (Eastern Rhodopes, South Bulgaria) were used in the study. The experimental samples, in the amount of 5-6 kg, were delivered for testing in the laboratory of "Biavin" Company – Plovdiv. The varieties and their origin were as follows:

- White wine varieties:

Dimyat – Village of Dimitrovche, Svilengrad Municipality, Haskovo Region
Red Misket – Village of Dimitrovche, Svilengrad Municipality, Haskovo Region
Tamyanka (1) – Village of Shishmanovo, Harmanli Municipality, Haskovo Region
Tamyanka (2) – Village of Susam, Mineralni Bani Municipality, Haskovo Region

- Red wine varieties:

Pamid (1) – Town of Lyubimets, Haskovo Region
Pamid (2) – Village of Kolarovo, Harmanli Municipality, Haskovo Region
Gamza – Village of Shishmanovo, Harmanli Municipality, Haskovo Region
Mavrud (1) – Town of Lyubimets, Haskovo Region
Mavrud (2) – Village of Shishmanovo, Harmanli Municipality, Haskovo Region
Mavrud (3) – Village of Susam, Mineralni Bani Municipality, Haskovo Region

The chemical composition of grapes for laboratory analysis is presented in Table 1. The content of basic chemical indicators – sugars, total acids and pH was analysed [28].

Table 1. Chemical composition of grapes from the experimental samples.

Grape variety	Indicators		
	Sugars g/l	Total acids g/l	pH
White varieties			
Dimyat	161.00	4.57	3.41
Red Misket	198.00	3.50	3.84
Tamyanka (1)	222.00	4.16	3.88
Tamyanka (2)	236.00	5.49	3.90
Red varieties			
Pamid (1)	210.00	3.71	3.99
Pamid (2)	205.00	2.64	3.96
Gamza	189.00	4.97	3.68
Mavrud (1)	184.00	4.32	3.84
Mavrud (2)	190.00	5.05	3.67
Mavrud (3)	181.00	6.87	3.52

2.1.2 Alcoholic fermentation

The grapes from experimental samples were processed under the conditions of microvinification. The grapes were first destemmed into containers cleaned with ethanol, then crushed and transferred into brand new 5 l PET vessels. The grape pomace was homogenized and sulphated with a 5% solution of H_2SO_3 (50 mg/l SO_2) before starting the spontaneous alcoholic fermentation at a temperature of 25°C. The course of the process was monitored daily, for 13 days, by refractometer, after homogenization and mixing of the solid parts with the liquid phase. The change in dry

matter (%) and the end of the process were monitored until a constant value was established and held.

Upon the completion of the alcoholic fermentation in all experimental samples, the liquid phase was separated from the solid parts without pressing. The wines were transferred to clean PET vessels and stored for further research – isolation of the microorganisms present in the medium and study of their morphological and cultural characteristics.

2.2 Isolation of morphological types from spontaneously fermented experimental wines

After the appropriate dilutions in sterile physiological solution, inoculations were made on malt extract/agar complex medium (two replicates) and on malt extract/agar selective medium supplemented with 20 mg/l actidione. The Petri dishes were placed in a thermostat at a constant temperature of 27ºC to monitor the individual colonies growth.

From the established morphological types, according to Koch's method [21, 28, 29, 30], pure cultures were isolated – from the complex nutrient media on the 96th hour, and from the selective ones on the 168th hour. For the yeast strains, the isolation was carried out in test tubes with 10 ml of sterile grape juice, and for the bacteria in test tubes with 10 ml of sterile bacterial medium. The samples were kept in a thermostat at a temperature of 25°C for 5-7 days; thereafter they were subjected to laboratory tests. The collection of the isolated morphological types after their growth was placed in cold storage.

2.3 Morphological and cultural characteristics of newly isolated morphological types

The morphological characterization was done on 72-hour liquid cultures developed in sterile grape juice medium, and the cultural one – on 72-96 - hour colonies formed on grape juice-agar [28, 30].

2.4 Sporulation in the newly isolated yeast strains from spontaneously fermented experimental wines

The sporulation in the newly isolated yeast strains was studied by a classic method [28, 30, 31, 32] – a single colony growth on a complex solid nutrient medium, transfer of biomass to starved agar (agar without a carbon source), storage in a thermostat at a temperature of 25°C for 6 days and microscopy of a smear from the biomass.

3 Results and Discussion

3.1 Isolation of morphological types from spontaneously fermented experimental wines

On the complex nutrient medium, the microorganisms' growth began already on the 24-36th hour, and on the selective medium around the 96th hour, but only in some samples. Selective cultural medium with added actidione is most often used in studies to identify actidione-resistant microorganisms. This mainly refers to yeasts of *Brettanomyces* species, which are common in winemaking, but are defined as undesirable microflora. During their metabolism, they have an increased production of volatile phenols from cinnamic acids, which deteriorate the aroma of the wine [33, 34, 35, 36].

Figure 1 and Figure 2 show the colonies formed in two of the variants – Red Misket (village of Dimitrovche) and Tamyanka (village of Susam).

Fig 1. Colony growth from Red Misket sample.

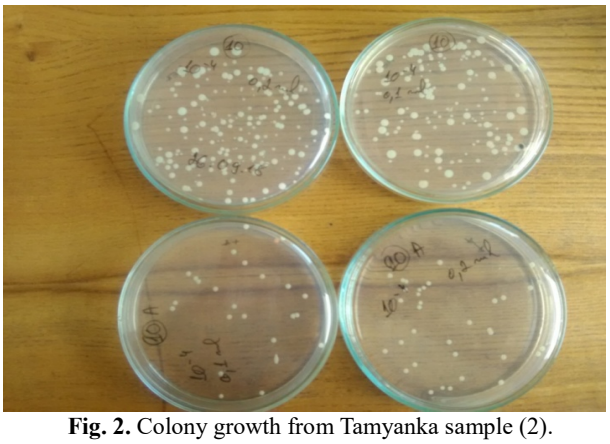


Fig. 2. Colony growth from Tamyanka sample (2).

Table 2 presents a detailed description of the grown individual colonies on complex and selective nutrient medium. After the investigation, a total of 38 morphological types were isolated – respectively 25 from complex media and 13 from selective media. In some cases, morphological types were isolated, differing only in the colour shade or the colony size. This approach was implemented in order not to miss an available morphological type.

From the data in Table 2, it could be seen that in almost all samples at the end of the process, the diversity of morphological types was small that was quite expected and logical.

Table 2. Morphological types, grown on complex and selective cultural media.

№	Source	Code	Colony description
1	Dimyat	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	Red Misket	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 (1)	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 (1)	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 (2)	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	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 (1)	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 (2)	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 (3)	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 (2)	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.

The presumably found microorganisms were markedly competitive and in the course of the process displaced the others presented on the grapes. It was likely these were the microorganisms that carried out the main part of the alcoholic fermentation, and with their metabolism had determined the chemical composition and sensory profile of the obtained experimental wines. More morphological types were established in the Tamyanka sample (2). That was probably due to the lack of strong antagonists, which allowed different types of microorganisms to participate in the process until its completion.

3.2 Morphological and cultural characterization of newly isolated morphological types from spontaneously fermented experimental wines

There were studies on the morphological and cultural properties of newly isolated strains of *Saccharomyces cerevisiae*, for making of regional wines from Regent variety [37, 38]. Under microscopy, the authors revealed a similar morphology in most strains – the cells were uniform oval to slightly elliptical. Their size varied from 3-5 to 6-9 μm. The cells were located in the medium singly and in small groups. The strains reproduced vegetatively by unilateral polar budding, rarely bilaterally. In a liquid nutrient medium (grape juice), the yeast developed uniformly, causing active fermentation with turbidity of the medium, gas releasing and foam formation. At the end of the process, some strains formed a light ring on the surface, and relatively compacted sediments at the bottom. On a solid nutrient medium, they formed colonies of the same type – entire, smooth, dome-shaped convex, semi-shiny, cream to light beige, pasty.

In the present study, a morphological characterization of the microorganisms isolated from pure cultures of spontaneously fermented wines was made. They were identified as yeast or bacteria respectively. Their shape, size, arrangement of cells in the medium and method of vegetative propagation were determined. The results are presented in Table 3. The cell sizes of the isolated yeast strains were similar, ranging from 3-6 μm in width to 4-10 μm in length.

Table 3. Morphological and cultural characteristics of the isolated strains.

№	Source	Code	Colony description	Microorganism type yeast/bacterium	Cell morphology
1	Dimyat	1-1	Large, smooth, semi-glistening, dome-shaped with a dimple, light cream-coloured, pasty, entire	Y	Oval-ellipsoid cells, individually arranged 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, arranged in pairs in the medium, tremulous
4	Red Misket	2-1	Large, smooth, semi-glistening, dome-shaped with a dimple, light cream-coloured, pasty, entire	Y	Spherical-oval cells, individually arranged 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, arranged in pairs in the medium, tremulous
7		2-4-A		B	
8	Mavrud (1)	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 arranged 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, arranged in pairs in the medium, tremulous
11	Pamid (1)	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 arranged 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 arranged 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, arranged in pairs in the medium, tremulous
15	Pamid (2)	5-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Oval-ellipsoid cells, individually arranged 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, arranged mainly in fours in the medium, immobile
18	Gamza	6-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Oval-ellipsoid cells, individually arranged 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 arranged 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, arranged in pairs and in chains in the medium, immobile
22	Tamyanka (1)	7-1	Medium to large, smooth, semi-glistening, dome-shaped, light beige, pasty, entire	Y	Ellipsoid-oval cells, individually arranged 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, arranged in pairs 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, arranged in pairs in the medium, tremulous
26	Mavrud (2)	8-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Oval to ellipsoid cells, individually arranged 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, arranged in pairs in the medium, tremulous
29		8-4-A	Very small, smooth, pearlescent, transparent, glistening	B	Coccoid shape of the cells, arranged in pairs and in chains in the middle, immobile
30	Mavrud (3)	9-1	Large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Spherical-oval to ellipsoid cells, individually arranged 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 (2)	10-1	Medium to large, smooth, semi-glistening, dome-shaped, cream-coloured, pasty, entire	Y	Ellipsoid-oval cells, individually arranged 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 arranged in the medium, smaller than 10-1; vegetative propagation by budding: unilateral, polar and a little to the side

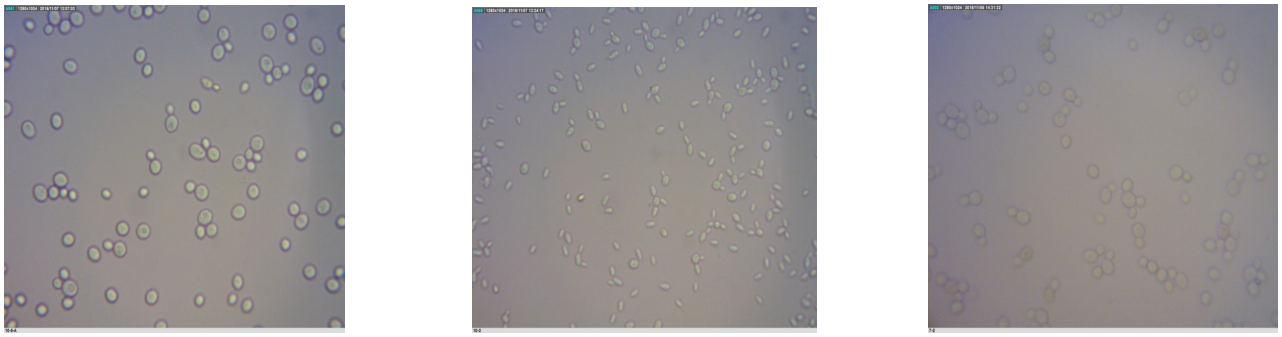
36		10-3	Small, cream-coloured to light beige, semi-glistening, smooth, with a dimple, entire	Y	Oval-spherical cells, larger than 10-1, individually arranged in the medium; vegetative propagation by budding: polar, unilateral
37		10-4-A	Small, beige to light brown, strongly glistening, transparent in the periphery, smooth, dome-shaped	B	Coccoid to ellipsoid shape of the cells, arranged in pairs 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 arranged in the medium; vegetative propagation by budding: polar, unilateral

Note: Y: yeast, B: bacteria

Microscopic images of some of the studied yeast strains are presented in Figure 3.

In most cases, yeast cells were spherical to slightly elliptical, located singly in the medium, reproduced vegetatively, by budding, most often unilaterally, polar (Figure 3a). In some strains, the cells were much smaller, elongated and slightly pointed (Figure 3b), and in some places multifaceted budding was observed (Figure 3c). In many cases, the morphological and cultural characterization found strong similarity or identity in the morphological types isolated as different. The size of the colonies was greatly influenced by the density of the inoculum, and the colour by the cultural age.

The yeast growth on a liquid culture medium, grape juice, was similar. The propagation started around the 18-24th hour, the medium was slightly turbid, individual gas bubbles were released. At the 36th hour, the medium was very turbid and foamy, 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 was clarified for different periods and to varying degrees with the different strains.



a) strain *10-5* b) strain *10-2* c) strain *7-2*

Fig. 3. Native microscopic preparation of part of the isolated yeast strains.

The growth on solid media was also similar. Colony sizes and colour shades varied slightly between individual strains.

In the microscopic characterization of the bacteria, cocci, slight ellipses and more elongated ellipses were most frequently observed. Propagation was by division everywhere. In some of the cultures the arrangement of cells was in pairs (10-4-A; 7-4-A), in others short chains of elliptical cocci were observed (8-4-A; 6-4-A), and in some tetrads (5-3-A; 7-3-A) had also been established.

The bacteria growth in a liquid cultural medium was similar and very uniform – poor development, almost no turbidity of the medium, no significant sediment was deposited, light veils were formed, visible when the test tube was stirred, in some cases a slight film was formed on the surface of the liquid (8-3-A; 10-4-A).

The bacterial colonies were very small, less than 1 mm in diameter, transparent, slightly slimy, highly shiny, some were pearly in colour (6-4-A; 8-4-A) and others were light beige (8-3-A; 10-4-A).

The obtained results for the morphological and cultural properties of the studied microorganisms were consistent with those identified by other authors who carried out similar studies [9, 11, 13]. In one of them, researchers isolated 86 local strains of *Saccharomyces cerevisiae* belonging to different physiological races and characterized them according to their resistance to SO_2, killer phenotype, growth at high temperature, and foam formation during fermentation [11]. Some authors [30] studied the morphological and physiological properties of yeast strains and found that all cultures had similar morphology with oval cells to slightly elliptical shape. The ability of strains to ferment sugars was determined. The strains fermented glucose, fructose, galactose, saccharose, maltose and 1/3 raffinose so all studied yeasts were related to the

species *Saccharomyces cerevisiae*. Other scientists reported the morphology, colour and texture of the colonies of the different strains on yeast peptone dextrose agar [16]. The colonies of *Saccharomyces cerevisiae* were described as umbonate, cream-colored, smooth/opaque; of *Hanseniaspora uvarum* as raised, cream-colored, smooth/glossy; of *Pichia fermentans* as convex, cream-colored, wrinkled; of *Torulaspora delbrueckii* as convex or umbonate, cream-colored, smooth/opaque. The strains belonging to the *Metshnikowia* genus exhibited biomass turning red during the prolonged incubation time. Studies were conducted on the morphological and cultural characteristics of different isolates of *Saccharomyces* and non-*Saccharomyces* species and the researchers found that the colour intensity of the yeast colonies was denoted by 1-white, 2-cream, 3-light brown, 4-brown, 5-dark and 6-black varied from smallest to largest. More isolates had a high flocculation capacity [3]. Other authors also observed microscopically the yeasts morphological characteristics and described that the cells shape were approximately spherical or oval-shaped, without pseudohyphae or spores. The morphology showed single, opposite or group single-terminal or double terminal budding [17]. Some scientists in their studies described colour of yeasts colonies varied from white, beige, pink and various shades of green to light brown. There was also difference in their shape – most were smoothly curved and some had a reduced margin or convex apex [21].

3.3 Sporulation in newly isolated yeast strains from spontaneously fermented experimental wines

A single colony growth on a complex solid nutrient medium and transfer of biomass on starved agar (agar without a carbon source) was monitored at 25°C for 6 days. Under these conditions, vegetative propagation of yeast is impossible due to the lack of nutrients. In such an environment, the cells cannot develop further and begin to sporulate [19, 31, 32]. The main focus of the present study was the isolation of wine yeasts for carrying out alcoholic fermentation. The sporulation as a physiological process directly related to their survival and adaptation under unfavourable conditions, has significance only for the taxonomy, classification and more complete characterization of the strains. Bacteria were identified as accompanying microflora, normally present in spontaneously fermenting grape must. Bacterial cells form spores only under optimal conditions. Due to the low pH values in wine (2.8 – 4.0), spore-forming bacteria do not develop [20].

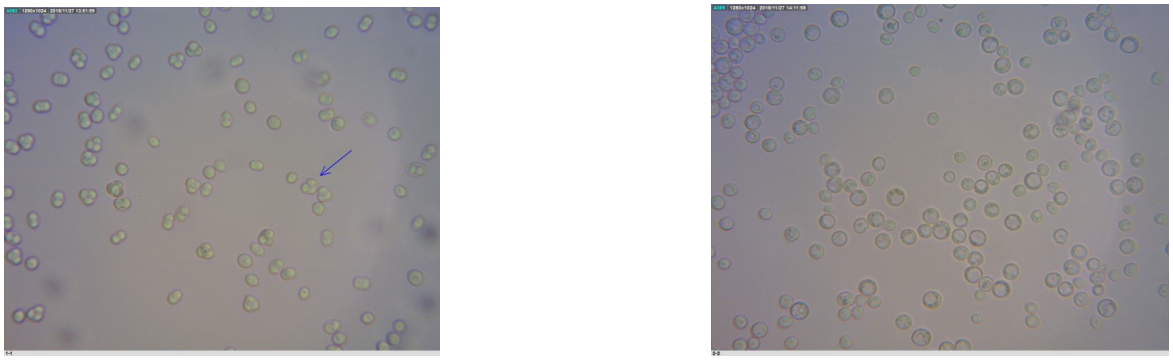
The results of biomass smear microscopy for sporulation in the newly isolated yeast strains in our study are presented in Table 4. Almost all newly isolated yeast strains formed spores. Most often relatively abundant to abundant – between 50-60% and 70-80% of the cells sporulated. Only strain 2-2 showed no sporulation, and strain 10-2 showed very little sporulation. In all other strains, the spore formation was without prior copulation, in the asci most often 2 to 4 spores were observed, spherical to oval in shape. In some cases, a slight deformation of the ascus was observed.

Table 4. Sporulation in the newly isolated yeast strains.

№	Source	Code	Microscopic results, spore formation (*without previous copulation)
1	Dimyat	1-1	Abundant spore formation, mainly 3 spores per ascus, *
2		1-2	Abundant spore formation, mainly 3 spores per ascus, *
3		2-1	Abundant spore formation, mainly 3, rarely 4 spores per ascus, *
4	Red Misket	2-2	Very poor or did not form spores on agar without a carbon source
5	Mavrud (1)	3-1	Rarely forms spores, 1 – 2 spores per ascus, *
6		3-2	Very rarely forms spores, 1 – 2 spores per ascus, *
7		4-1	Very rarely forms spores, 1 – 2 spores per ascus, *
8	Pamid (1)	4-2	Fairly abundant spore formation, 1 – 2 spores per ascus,*
9		4-3	Abundant spore formation, 3 – 2 spores per ascus, *
10	Pamid (2)	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		6-1	Abundant spore formation, 2 – 4 spores per ascus, *
13	Gamza	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		7-1	Abundant spore formation, 2 – 4 spores per ascus, *
16	Tamyanka (1)	7-2	Abundant spore formation, 2 – 4 spores per ascus, *
17		7-3	Abundant spore formation, most often 3 spores per ascus,*
18	Mavrud (2)	8-1	Fairly abundant spore formation, 2 – 3 spores per ascus, *
19		8-2	Abundant spore formation, from 2 – 4 spores per ascus, *
20		9-1	Fairly abundant spore formation, 2 – 3 spores per ascus, *
21	Mavrud (3)	9-2	Abundant spore formation, 2 – 3 spores per ascus, *
22		9-3	Fairly abundant spore formation, from 2 – 4 spores per ascus, *
23		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	Tamyanka (2)	10-3	Abundant spore formation, from 2 – 4 spores per ascus, *
26		10-5	Medium-abundant spore formation, 2 – 3 spores per ascus, *

Microscopic images of some of the studied strains are presented in Figure 4.



a) strain *1-1* b) strain *2-2*

Fig. 4. Microscopic image of sporulation.

These results were like those found in similar studies by other authors. Some of them [37, 38] observed sporulation without prior copulation in about 50–60% of the cells, forming 2–3 ascospores per ascus in newly isolated *Saccharomyces cerevisiae* strains. Others [30] studying the sporogenesis of *Saccharomyces cerevisiae* found difference of sporulation degree and that all strains produced spores during a period of 7-8 days.

4 Conclusion

As a result of spontaneous alcoholic fermentation of grape pomace from 10 experimental samples of grapes, a total of 38 morphological types were isolated, of which 26 yeast strains and 12 bacterial strains. Morphological and cultural identification were done to all of them. In most cases, yeast cells were spherical to slightly elliptical, located singly in the media. They reproduced by budding, most often unilaterally, polar. The cell sizes of the isolated yeasts were similar and ranged from 3-6 µm to 4-10 µm in length. The growth of the different strains of yeast and bacteria in liquid and on solid media was similar. A sporulation process had also been observed in yeast. Almost all newly isolated strains formed spores without prior copulation, as about 50-60% and 70-80% of the cells sporulated.

Acknowledgements

This research was carried out under the project "Developing identity site"/DIONYSOS, on yield, Subsidy soil and contract B2.6c.04/01.11.2017 with the financial support of the INTERREG V-A GREECE - BULGARIA 2014-2020 COOPERATION Program, Co-funded by the European Regional Development Fund and National funds of Greece and Bulgaria. Total budget: EUR 735 778.70 (https://dionysosvine.eu/en/).

References

1. A. Barata, M. Malfeito-Ferreira, V. Loureiro. The microbial ecology of wine grape berries. Int. J. Food Microbiol. **153**, 243-259 (2012). https://doi.org/10.1016/j.ijfoodmicro.2011.11.025

2. C. Bezerra-Bussoli, M. A. Baffi, E. Gomes, R. Da Silva. Yeast diversity isolated from grape musts during spontaneous fermentation from Brazilian winery. Curr. Microbiol. **67**(3), 356–361 (2013). https://doi.org/10.1007/s00284-013-0375-99

3. M. T. Ivkovikj. Isolation of indigenous yeasts from different types of red grapes and determination of oenological characteristics of wine. KIJ **47**(3), 437-442 (2021).

4. J. Sabate, J. Cano, B. Esteve-Zarzoso, J. M. Guillamon. Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. Microbiol. Res. **157**, 267-274 (2002). https://doi.org/10.1078/0944-5013-001633

5. M. A. Baffi, C. dos Santos Bezarra, M. Arevalo-Villena, A. I. Briones-Perez, E. Gomes, R. Da Silva. Isolation and molecular identification of wine yeasts from a Brazilian vineyard. Ann. Microbiol. **61**, 75-78 (2011). https://doi.org/10.1007/s13213-010-0099-z

6. E. Jankura, L. Piknova, J. Lopašovska. Identification and technological characteristics of yeast strains from vineyards in Slovakia. J. Food Nutr. Res. **59**(3), 241-249 (2020).

7. C. P. Kurtzman, J. W. Fell, T. Boekhout. Definition, classification and nomenclature of the yeast. The Yeasts (Elsiver, 2011), Chapter 1, pp. 3-5. https://doi.org/10.1016/B978-0-444-52149-1.00001-X

8. J. A. Hernandez-Garcia, E. De-la-Vega-Camarillo, L. Villa-Tanaca, C. Hernandez-Rodriguez. Yeast taxonomy. Yeasts: From Nature to Bioprocesses Mycology: Current and Future Developments (Bentham Science Publishers Pte. Ltd. Singapore, 2022), Chapter 3, pp. 58-72. https://doi.org/10.2174/9789815051063122020005

9. S. Rainieri, I. S. Pretorius. Selection and improvement of wine yeasts. Ann. Microbiol. **50**(1), 15-31 (2000).

10. M. Kačaniova, L. Hleba, J. Pochop, M. Kadasi-Horakova, M. Fikselova, K. Rovna. Determination of wine microbiota using classical method, polymerase chain method and step one real-time PCR during fermentation process. J. Environ. Sci. Health, Part B, **47**(6), 571-578 (2012). https://doi.org/10.1080/03601234.2012.665750

11. J. A. Regodon, F. Perez, M. E. Valdes, C. De Miguel, M. Ramirez. A simple and effective procedure for selection of wine yeast strains. Food Microbiol. **14**, 247-254 (1997).

12. O. Csernus, A. Pomazi, I. Magyar. Isolation, characterization and selection of wine yeast strains in Etyek-Buda wine district. Acta Aliment. **43**(3), 489-500 (2014). https://doi.org/10.1556/AAlim.2014.1111

13. P. Zabukovec, N. Čadez, F. Čuš. Isolation and identification of indigenous wine yeasts and their use in alcoholic fermentation. FTB **58**(3), 337 (2020). https://doi.org/10.17113/ftb.58.03.20.6677

14. H. Pavlović, V. Petravić Tominac, D. Velić, T. Madarević Pavetić, V. Zechner-Krpan, N. Velić. Application of wine yeast starter cultures in the production of grape and fruit wines. FERM. **11**(4), 228 (2025). https://doi.org/10.3390/fermentation11040228

15. M. Sunyer-Figueres, D. Fernandez-Vazquez, C. Cuesta-Marti, I. Horcajo-Abal, C. Sanchez-Mateos, A. Domenech, E. Nart, V. A. Castillo-Olaya, I. Andorra, M. Puxeu. Impact of grape harvest time on wild yeast biodiversity and its influence on wine fermentation. Microorganisms **13**, 2836 (2025). https://doi.org/10.3390/microorganisms13122836

16. R. Sidari, K. Ženišova, B. Tobolkova, E. Belajova, T. Cabicarova, M. Bučkova, A. Puškarova, M. Plany, T. Kuchta, D. Pangallo. Wine yeast selection: laboratory characterization and protocol review. Microorganisms, **9**, 2223 (2021.). https://doi.org/10.3390/microorganisms9112223

17. X. Yu, J. Xu, Y. P. Li, X. P. Li. Isolation and screening of wine yeasts from grapes in Yalu river valley China for fermentation performance. Am. J. Biochem. Biotechnol. **17**(3), 338-345 (2021). https://doi.org/10.3844/ajbbsp.2021.338.345

18. I. Piekarska, J. Rytka, B. Rempola. Regulation of sporulation in the yeast *Saccharomyces cerevisiae*. Acta Biochim. Pol. **57**(3), 241-250 (2010). PMID 2084229

19. K. M. Thomasson, A. Franks, H. Teotonio, S. R. Proulx. Testing the adaptive value of sporulation in budding yeast using experimental evolution. Evolution **75**(7), 1889-1897 (2021). https://doi.org/10.1111/evo.14265

20. E. A. Hutchison, D. M. Miller, E. R. Angert. Sporulation in bacteria: beyond the standard model. Microbiol. Spectr. **2(**5), 26104376 (2014) https://doi.org/10.1128/9781555819323.ch4

21. J. Lakatošova, L. Priesolova, E. Jankura. Isolation and identification of yeasts isolated from grapes and spontaneously fermented wines. 37th World Congress of Vine and Wine, Mendoza, Argentina, November (2014).

22. J. X. Xue, Y. W. Xu, Y. Yang, Y. L. Liu. Investigation on the tolerance of wild *Saccharomyces cerevisiae*. Liq. Mak. Sc. Technol. **6**, 45 (2007).

23. X. L. Xu, G. L. Feng, H. W. Liu, X. F. Li, G. L. Zhao, X. L. Xiao. Isolation, identification and control of osmophilic spoilage yeasts in sweetened condensed milk. Afr. J. Microbiol. Res. **8**(10), 1032-1039 (2014). https://doi.org/10.5897/AJMR2013.6203

24. B. Esteve-Zarzoso, A. Gostincar, R. Bobet, F. Uruburu, A. Querol. Selection and molecular characterization of wine yeasts isolated from the 'El Penedes' area (Spain). Food Microbiol. **17**(5), 553-562 (2000). https://doi.org/10.1006/fmic.2000.0347

25. M. L. Raymond Eder, C. Reynoso, S. C. Lauret, A. L. Rosa. Isolation and identification of the indigenous yeast population during spontaneous fermentation of Isabella (*Vitis labrusca* L.) grape must. Front. Microbiol. **8**, 532 (2017). https://doi.org/10.3389/fmicb.2017.00532

26. H. Zhuang, T. Feng. Isolation, identification, and application of yeast strain from the local ecosystem of Summer Black vineyard. J. Food Process. Preserv. **46**(5), (2022). https://doi.org/10.1111/jfpp.16341

27. R. S. Rădoi-Encea, V. Pădureanu, C. F. Digută, M. Ion, E. Brinduşe, F. Matei. Achievements of autochthonous wine yeast isolation and selection in Romania – a review. FERM. **9**(5), 407 (2023). https://doi.org/10.3390/fermentation9050407

28. T. Ivanov, S. Gerov, A. Yankov, G. Bambalov, T. Tonchev, D. Nachkov and M. Marinov. Practicum in wine technology, (Publ. House "Hristo G. Danov", Plovdiv, 1979), pp. 403-469.

29. F. Ilieva, S. Kostadinović Veličkovska, V. Dimovska, H. Mirhosseini, H. Spasov. Selection of 80 newly isolated autochthonous yeast strains from the Tikveš region of Macedonia and their impact on the quality of red wines produced from Vranec and Cabernet Sauvignon grape varieties. Food Chem. **216**, 309-315 (2017). https://doi.org/10.1016/j.foodchem.2016.08.049

30. F. Ilieva, S. Kostadinović Veličkovska, V. Dimovska, H. Mirhosseini, H. Spasov. Isolation of *Saccharomyces cerevisiae* yeast strains from Macedonian "Tikveš" wine-growing region and their impact on the organoleptic characteristics of Vranec and Cabernet Sauvignon wines. Res. J. Biotech. **14**(6), 100-110 (2019).

31. S. Piccirillo, S. M. Honigberg. Sporulation patterning and invasive growth in wild and domesticated yeast colonies. Res. Microbiol. 161(5), 390-398 (2010). https://doi.org/10.1016/j.resmic.2010.04.001

32. A. M. Neiman. Sporulation in the budding yeast *Saccharomyces cerevisiae*. Genetics **189**(3), 737-765 (2011). https://doi.org/10.1534/genetics.111.127126

33. B. D. Smith, B. Divol. *Brettanomyces bruxellensis*, a survivalist prepared for the wine apocalypse and other beverages. Food Microbiol. **59**,

161-175 (2016). https://doi.org/10.1016/j.fm.2016.06.008

34. C. Berbegal, G. Spano, M. Fragasso, F. Grieco, P. Russo, V. Capozzi. Starter cultures as biocontrol strategy to prevent *Brettanomyces bruxellensis* proliferation in wine. Appl. Microbiol. Biotechnol. **102**(2), 569-576 (2017). https://doi.org/10.1007/s00253-017-8666-x

35. M. Malfeito-Ferreira. Two decades of "Horse Sweat" taint and *Brettanomyces* yeasts in wine: where do we stand now? Beverages **4**(2), 32 (2018). https://doi.org/10.3390/beverages4020032

36. P. Branco, R. Coutinho, M. Malfeito-Ferreira, C. Prista, H. Albergaria. Wine spoilage control: impact of Saccharomycin on *Brettanomyces bruxellensis* and its conjugated effect with sulfur dioxide. Microorganisms **9**(12), 2528 (2021). https://doi.org/10.3390/microorganisms9122528

37. H. Spasov, D. Hristev, N. Blagoeva, N. Stoyanov, P. Mitev, I. Tchobanov, P. Stefenova. Comparative study of newly yeast strains from different regions of Bulgaria. Sc. Works UFT, Food Sc., Eng. Technol. **LIX**, 397-402 (2012).

38. H. Spasov, Y. Chobanov, N. Blagoeva, P. Zapryanova, Zh. Raychevska, A. Mosengova. Selection of newly isolated yeast strains for the production of regional wines of the Regent variety. J. Mt. Agric. Balk. **20**(4), 107-121 (2017).