

Identification and characterization of some yeast strains from the Stefanesti – Arges Vineyard

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ABSTRACT

The purpose of the present work was to identify and characterize several yeast strains useful in fermentation process and present in the Stefanesti-Arges vineyards. The biological material used was freshly squeezed grape must from the white wine grape cultivar, 'Fetească regală' one of the most widespread and appreciated cultivars for wine in the Arges region. In this study is described the procedure of obtaining the pure isolates and are presented the macroscopic and microscopic characteristics of these isolates retained on the culture medium. By microscopy and molecular analyses was confirmed that the selected isolates in pure culture belongs to the genus *Saccharomyces*. The morphological characteristics, complemented by the molecular ones of the PCR amplification of the region ITS1-5,8S -ITS2, as well as the analysis of the "killer" profile of the selected strains, proved that the four isolates obtained and analyzed are identical and belong to the species *Saccharomyces cerevisiae*.

Keywords: epiphytic microflora, grapes, pure culture, ITS-RFLP, *Saccharomyces*.

INTRODUCTION

In the taxonomy field of viticultural and wine microflora, the reference study is the one prepared by Lodder (1970). Since then, the taxonomy of the levurian microflora has been the subject of a significant number of research papers, such as that one written (Gayon *et al.*, 2006). The studies regarding grapes and wine microflora is oriented towards the identification of new yeast strains and species that can be useful in the fermentation processes, or can contribute in solving the shortcomings that could appear in the production flows (González, 2007). On the grapes surface, in all phases of ripening, especially at grape harvest season, different categories of microorganisms are found. A wide cultivar of epiphytic microorganisms, including bacteria, yeasts and filamentous fungi, is found on grapes (Papadopoulou *et al.*, 2022). Through the technological process of crushing, all these inevitably end up in the mash. Due to the rich nutritional composition of the grapes must, epiphytic microorganisms can proliferate and change must fermentation, causing either desired or unwanted changes. Yeasts studies have made significant progress in the recent years, especially in the field of genetics and cell biology. Characterization of individual species of yeasts by morphological, physiological and biochemical criteria is considered a first priority for winemaking (Lakatošová *et al.* 2014; Raymond Eder *et al.* 2017). These

contributed to the improvement of fermentative processes of winemaking (Molinet and Cubillos, 2020). Kántor and Kačániová (2015) pointed out that the grape surface is a generally unstable living environment for microorganisms. Maicas (2020) describes how wine is the result of the interaction of several species and strains of yeasts.

The aim of this study is to identify and characterize endemic yeast strains from the Stefanesti-Arges vineyard that could be used in the fermentation process for winemaking.

MATERIALS AND METHODS

Grapes of 'Fetească regală' cultivar were used to isolate the epiphytic yeasts analyzed in this study. Grapes were harvested from the Germoplasm Collection of NRDIBH Stefanesti-Arges, in September 2021. This grape cultivar for white wine was preferred as it is one of the most well-known and widespread vine cultivars in the Arges wine-growing area, and as well in Romania.

Yeast isolation and characterization

In order to isolate new strains of epiphytic yeasts able to start the natural fermentation process of grapes' must, freshly squeezed grapes were used.

Healthy grapes were crushed in sterile mortar and pestle sets. The resulted mash was let to stay in the laminar flow for 20 minutes for infusion. Must samples were processed by the serial decimal dilutions' technique (Domerg, 1956) and plated on Potato-Dextrose-Agar with chloramphenicol (PDA/ch) culture medium (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Cultures were incubated in electrical thermostat incubator (CLN 53 Smart, Pol-EKO) at 28°C for 48-72 hours. Resulting yeast colonies were selected based on their morphology and new strains were purified using YPG medium (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and the streak plate method (Cappuccino and Sherman, 2004).

The cells' morphology of newly isolated strains was microscopic characterized using the "OPTIKA" digital binocular microscope, coupled with the B-290TB "OPTIKA" tablet according to Pintilie (2011) protocol for yeasts analysis.

Killer character was also evaluated for each newly isolated strain. To highlight the killer character the biosensor sensitive strain *S. cerevisiae* 17/17 was used. This strain was inoculated on the surface of specific medium for killer testing (phosphate citrate buffer 0.1M, pH4.8; glucose 2%; yeast extract 1%; peptone 1%, agar 2%; methylene blue 0.03-0.07%), while the studied yeast isolates were inoculated in streaks. Two reference strains of *S. cerevisiae*, SMR4 and X208, were used as positive controls strains with killer effect.

Resistance to killer toxins was also evaluated. For this, the newly isolated yeasts were inoculated on the surface of killer testing media, while all the other isolated yeasts strains together with the references were inoculated in streaks. Incubation was made at room temperature, as the killer protein is thermolabile.

Total DNA isolation

Total DNA was extracted from fresh yeast cultures. DNA isolation and purification were performed with the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) following the manufacturer protocol. The quantity and quality of total genomic DNA was performed by spectrophotometric method using a SpectraMax QuickDrop Micro-Volume Spectrophotometer nanodrop machine (Molecular Devices, Cambridge, UK).

PCR technique for amplifying the ITS region

The amplification of the ITS1-5.8S-ITS2 region was carried out using the universal primers ITS1(5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR reactions were carried out using the Mango Taq™ DNA Polymerase kit from Meridian Bioline (Meridian Life Sciences Inc., Memphis, Tennessee, USA), in 50 µl reaction volume, containing: 1X Buffer CL, 1.5 mM MgCl₂, 0.2mM dNTPs, 0.5 µM of each primer, 0.25 U of MangoTag DNA Polymerase and a maximum of 20 ng of genomic DNA. The

amplification program (Table 1) was carried out in an Eppendorf 5331 MasterCycler Gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany), as described below.

Table 1. PCR program for amplification

PCR Steps	Cycles	Parameters
Initial denaturation	1	94°C, 3min
Denaturation	35	94°C, 1min
Annealing		55.5°C, 1min
Elongation		72°C, 2min
Final elongation	1	72°C, 7min

Restriction fragment length polymorphism (RFLP)

ITS-PCR products were enzymatically cleaved with three restriction enzymes: *HaeIII*, *HinfI*, *HhaI*, (Thermo Fisher Scientific, Vilnius, Lithuania) according to Esteve-Zarzoso *et al.* (1999). Enzymatic digestion was performed in an Eppendorf 5331 MasterCycler Gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany), for 2 hours at 37°C, according to the indications provided by the manufacturer for the reaction mixture.

Gel electrophoresis

The ITS-PCR amplicons and the digested PCR products were examined by 1%, and 2% respectively, agarose gel electrophoresis (Promega Corporation, Madison, WI, USA) in 0.5X TBE buffer (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), supplemented with ethidium bromide (Sigma Chemical Co., Steinheim, Germany). The electrophoretic profiles were then analysed under UV light using the BioDoc-It Imaging System (Ultra-Violet Products Ltd., Cambridge, UK). To estimate the size of the DNA molecules that were separated based on their mobility in an electrical field were used DNA ladder of 100 bp (Thermo Scientific, Vilnius, Lithuania). The restriction fragments profiles were compared with the electrophoretic patterns of various yeast species presented by Esteve-Zarzoso *et al.* (1999), Sabate *et al.* (2002), and Baffi *et al.* (2010).

RESULTS AND DISCUSSIONS

Isolated yeasts

The microbial load associated to freshly crushed grapes of 'Fetească regală' was evaluated at 10⁶ CFU/ml. The semi-selective PDA/ch sustained the growth of yeasts and molds, as well as some bacteria resistant to chloramphenicol (Figure 1 a). Colonies showing round, risen or slightly domed, opaque, dull, with white to cream color were selected and purified (Figure 1 b). Four yeasts isolates were then obtained and named D1, D2, D3 and D4. Similar colony morphology was obtained on YPG agar medium (Figure 1 c). This method is mentioned to be more efficient than direct yeast isolation from undamaged grapes (Mercado *et al.*, 2007; Valero *et al.*, 2007) most probably due to the low density of fermentative *Saccharomyces* on the intact fruit skin compared to other microbial epiphytic colonizers, such as non-*Saccharomyces* yeasts and spores of filamentous fungi (Ükelgi, 2011).

Microscopic characteristics of newly isolated yeasts

Microscopy studies have been conducted compared to the reference strain *Saccharomyces cerevisiae* 17/17. The cells showed ovoid shape, many of which were in the budding stage. In Lugol's solution, vacuoles were revealed uncolored and large, inside cytoplasm, while glycogen granules were reddish-brown in color (Figure 2). The microscopic examination of obtained yeast cultures revealed that all the yeast strains used came from pure cultures. Cell morphology did not vary markedly from one strain to another, and cells showed ovoid shape. The measurements of yeast size cells showed small differences among the four isolates (Table 2).

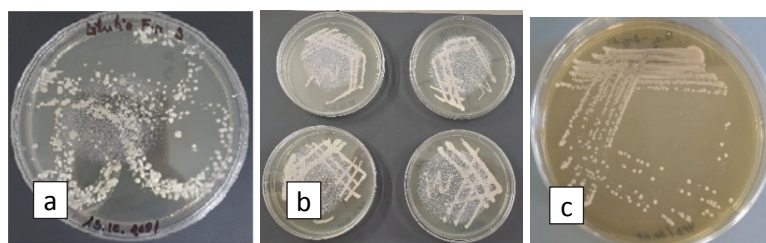


Figure 1. Yeast cultures in different stages of isolation and purification
a. in mixed cultures on PDA/ch, **b.** in pure cultures on PDA/ch, **c.** in pure culture on YPG agar

The microscopic examination of obtained yeast cultures revealed that all the yeast strains used came from pure cultures. Cell morphology did not vary markedly from one strain to another, and cells showed ovoid shape. The measurements of yeast size cells showed small differences among the four isolates (Table 2). It is known that the morphological characteristics of the yeasts present on the surface of the grape berries differ depending on the environmental conditions and the diameter of the cells varies on average between 3 and 4 μm (Walker *et al.* 2002). Our results proved that the four isolates have dimensions between 5.2 and 6.2 μm long and 4.7 and 5.1 μm wide. These measurements are in accordance to those mentioned by Milo and Phillips (2015), using light microscopy approaches and ranging between 4 μm in haploids and 6 μm in diploids cells. Also, these cell morphology size range are commonly found on single cells and one-budded mother cells of *Saccharomyces* yeasts (Aon *et al.*, 2018).

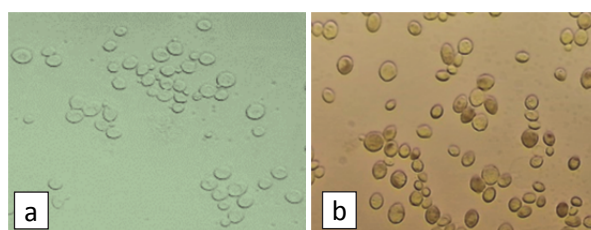


Figure 2 Microscopic images of yeast cells obtained in pure cultures
a. D1 new strains, **b.** *Saccharomyces cerevisiae* 17/17 reference strain

Table 2. Average dimensions of yeast cells grown on the PDA/ch medium after three days of incubation

Yeast strains	Cell size	
	Average length (μm)	Average width (μm)
D1 isolate	5.50 $\pm$ 0.42	5.10 $\pm$ 0.25
D2 isolate	5.25 $\pm$ 0.35	4.87 $\pm$ 0.28
D3 isolate	6.28 $\pm$ 0.35	5.04 $\pm$ 0.32
D4 isolate	5.75 $\pm$ 0.30	4.68 $\pm$ 0.30

All these macroscopic aspects of the cultures obtained on PDA/ch and YPD medium, as well as cytological aspects and the measurements strongly sustained that the yeast isolates belong to the genus *Saccharomyces*.

DNA concentration and purity

The results obtained and shown in Table 3, proved that the applied extraction method was efficient and were obtained eluted total DNA samples with high purities and in good quantities for further PCR reactions. The higher values than 1.8 for A260/A280 ratio and the lower values than 2.0 for A260/A230 ratio indicated a certain degree of contamination

with salts and carbohydrates of the extracted DNA. It is known that these types of contaminants could affect the efficiency of amplification during PCR reactions. With all total DNA samples were amplified properly the specific region of ITS 1-5,8S-ITS2, proving the efficiency of the applied extraction method and the good enough quality of the obtained eluted solutions.

Table 3. DNA concentration and purity

Yeast strains	Concentration <ng/ μ l>	Optical density ratios	
		A260/A280	A260/A230
D1	66	1.941	1.650
D2	48	1.920	1.714
D3	60	2.000	1.765
D4	74	1.956	2.312

PCR amplified ITS1-5,8S -ITS2 region

The amplified products obtained for ITS region using ITS1 and ITS4 primers were uniform and was revealed by a single band of 840 bp (Figure 3) for all the four isolates. According to White *et al.* (1990) and Arlorio *et al.* (1999) this band size is specific for isolates of *Saccharomyces* genus. Beside this confirmation, our results are similar to Cappello *et al* (2004) and proved the genetic homogeneity of the obtained isolates from 'Fetească regală' cv.

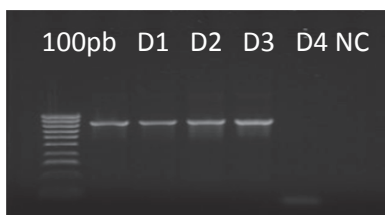


Figure 3. Electrophoretic pattern of yeasts ITS1-5,8S -ITS2 region obtained with ITS 1/4 primes set in 1% agarose gel; *Legend: **100pb** = DNA ladder; **D 1÷4** = isolated yeasts codes; **NC** = Negative control

ITS-RFLP profile of newly isolated yeast strains

A reliable identification of the yeast isolates was necessary. For this approach the ITS-PCR products were digested with three different restriction enzymes and the presence of certain bands, specific for region ITS1-5,8S -ITS2 were analysed (Esteve-Zarzoso *et al.*, 1999; Sabate *et al.*, 2002; Baffi *et al.*, 2010). Using *HaeIII*, *HinfI* and *HhaI* restriction enzymes, no variability was detected among the newly isolated strains (Figure 4). Moreover, the electrophoretic patterns and the restriction fragments' length of the digested ITS-PCR products confirmed *S. cerevisiae* affiliation of all four isolated strains (Table 4). Analysis of restriction profiles demonstrated that yeast species can be differentiated by combining the results obtained with each restriction endonuclease. The four obtained isolates revealed, or had the same or very similar restriction profile, and could be classified in the same group.

Killer profile of the new isolated *S. cerevisiae* strains

In winemaking processes, grapes natural microbiota can influence the fermentative process, thus contributing to the local specificity of the final product. Killer factor is highly important in selecting fermentative yeasts for winemaking processes. When selecting competitive yeast strains, among the current techniques, killer factor is highly important. Strain resistance to killer toxin, as well as strain potential for killer effect are both taken into consideration.

Yeast resistance to killer toxin enables the strain to survive and compete other related yeasts during fermentation. Therefore, this trait is desired in strains selected to perform controlled fermentation processes. As well, killer effect against other related yeasts spontaneously found in grapes natural microbiota is also important in wort fermentation. Killer yeasts are able to release a certain protein with toxic effect on other taxonomically related yeasts present in the same environment. This way killer yeasts are able to lyse sensitive yeasts cells. However, the producing strains are immune to this toxic protein, while the sensitive strains are killed in 48 hours (Figure 5). Such ability will give to the killer yeast the main role in conducting the fermentation process.

Table 4. RFLP analysis of the 5,8S-ITS region with three different restriction enzymes

Yeast strains	ITS-PCR product (pb)	Obtained restriction fragments (pb)			Identification
		<i>HaeIII</i>	<i>HinfI</i>	<i>HhaI</i>	
D1	840	320 + 230 + 180 + 130	360 + 120	360 + 330+130	<i>S. cerevisiae</i>
D2	840	320 + 230 + 180 + 130	360 + 120	360 + 330+130	<i>S. cerevisiae</i>
D3	840	320 + 230 + 180 + 130	360 + 120	360 + 330+130	<i>S. cerevisiae</i>
D4	840	320 + 230 + 180 + 130	360 + 120	360 + 330+130	<i>S. cerevisiae</i>

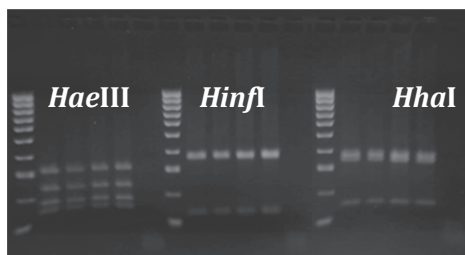


Figure 4. Restriction profiles of the ITS-5,8S region of yeast isolates after enzymatic digestion with *HaeIII* (left), *HinfI* (center) and *HhaI* (right). *Legend: *M* = 100pb DNA ladder; *D 1÷4* = yeast isolates; *NC* = Negative control

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When testing the newly selected isolates for sensitivity to killer proteins, no such clear zones of lysis were seen in the vicinity of the killer reference strains *S. cerevisiae* SMR4 and X208. However, the sensitive reference strain *S. cerevisiae* 17/17 was killed. Dead cells being invaded by the methylene blue (Figure 6). In *S. cerevisiae* three types of killer toxins are known K1, K2 and K3. However, K1 toxin is sensitive to high temperature, can be easily degraded by proteases, and remain stable at pH of 4.6-4.8. In wine, K2 type killer yeasts are

relevant, due to K2 toxin stability at a wider acidic pH, from 2.8 to 4.8 (Van Vuuren and Wingfield, 1986).



Figure 5. Lysis zone caused by the killer strain in the culture of sensitive strain *S. cerevisiae* 17/17.

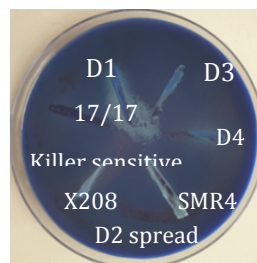


Figure 6. *S. cerevisiae* D2 new strain resistant to killer toxin released by *S. cerevisiae* SMR4 and X208 reference strains.

The K3 killer yeast were shown as mutants of K2 killer yeasts (Wingfield *et al.*, 1990). Beside competitiveness within the fermentation, K2 and K3 type of killer toxins revealed an important trait in food safety, as they are expressing antibacterial effects against a wide range of foodborne pathogens (Lakshmi and Mitra, 2020).

CONCLUSIONS

In order to obtain a characterization of the specific, autochthonous *S. cerevisiae* yeasts from the Stefanesti vineyards, which represent an important factor to promote natural fermentation and specific aroma of wines, from the cultures with mixed microbial load from the grape must of Fetească regală' cv. were obtained and analyzed four yeast isolates.

Microbiologic and ITS-RFLP genetic studies revealed these strains belong to *Saccharomyces cerevisiae*. These strains revealed to be resistant to the reference killer yeasts *Saccharomyces cerevisiae* SMR4 and X208 strains. Valuable strains for wine production, possessing resistance to killer yeast, could protect the wine fermentation against spoilage of contaminating killer yeasts. The results presented in this work were obtained for the first time with biological material from the Germplasm collection from Stefanesti-Argeș and represent an important step for continuing the investigations and deepening the knowledge regarding the obtaining and capitalization of the local yeast cultures in the winemaking processes.

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