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OBTAINING ACTIVE DRY YEASTS BIOMASS FOR THE PRODUCTION OF PIETROASA WINES

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Abstract

Consumers are increasingly demanding traditional beverages such as wines that are obtained through innovative technologies and that use indigenous yeasts to preserve the concept of "terroir". The paper presents the study performed for the exploitation the microbial diversity in the Pietroasa wine region in Romania and to obtain autochthones yeasts biomass. Yeasts were isolated from grapes and must from Feteasca regala FR) and Cabernet Sauvignon (CS) varieties cultivated at the Pietroasa Viticulture and Vinification Research and Development Station. Five yeast isolates were selected and identified by the MALDI-TOF, these belonging to the S. cerevisiae and non-Saccharomyces genera. By using these yeast isolates, active dry biomass was obtained through fermentation on a substrate based on sterilised diluted must (from the Feteasca regala and Cabernet Sauvignon varieties), synthetic sterilised media, followed by freeze-drying. Subsequently, the active dry biomass was used to obtain wines, using two types of nutrients: ET (Energyvin Thiols) and NO (Nutristart® ORG). All studied yeast isolates showed a promising potential for obtaining white wine from Feteasca regala grapes and rosé wine from the Cabernet Sauvignon grapes variety.

Key words*: non-Saccharomyces yeast, grapes, Pietroasa winery, winemaking.*

INTRODUCTION

To isolate and identify yeasts, a combination of microbiological culture-based approaches (*S. cerevisiae* and *non-S. cerevisiae*) is used in winemaking at laboratory level. Grape vines and grape musts have complex, locally specific microflora, among which yeast species may be responsible for spontaneous alcoholic fermentation (Bougreau et al., 2019). In addition, it is known that *Saccharomyces cerevisiae* is the main microorganism responsible for the alcoholic fermentation of wine (Javier Alonso-del-Real et al., 2017). Our research team selected and identified *S. cerevisiae* and *non-S. cerevisiae* yeast isolates from Pietroasa vineyard to obtain indigenous active dry yeast for winemaking. Five isolated yeasts have potential oenological interest and our research proved that one of them, non-*Saccharomyces* yeast with potential in must acidification, was recovered from the vineyard environment (Bougreau et al., 2019). *L. thermotolerans* has been shown to improve wine quality while naturally acidifying the wine in co-inoculation with *S. cerevisiae* (Comitini, 2011; Gobbi et al., 2013; Kapsopoulou et al., 2007). *Candida railenensis* strains were isolated from Slovak wines (Brežná et al., 2010; Drumonde-Neves et al., 2017; Cioch-Skoneczny, 2019). Based on the experience of Ramírez and Velázquez (2018), some strains of *T. delbrueckii* can fully dominate and complete the crushed grape fermentation to reach above 14% vol. alcohol, which is a very high alcohol content for non-*Saccharomyces* yeast

fermentation. Antoce and Cojocaru (2015) studied the obtaining of wines of Feteasca regala variety by inoculation and co-inoculation with several commercially available wine yeasts.
Our study was conducted on two grape varieties: Feteasca Regala (FR) and Cabernet Sauvignon (CS), from the Pietroasa Viticulture and Vinification Research and Development Station from the University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania (USAMVB). It is known that the Pietroasa Winery operated for a long time as one of the best equipped wineries in Romania, benefiting, since 1940, from a winemaking center equipped since 2018 with modern equipment that makes possible the industrial scale-up of wines obtained at pilot level. From the point of view of wine production, Romania produces approximately 3.1% of the wine of the world based on the available data for the year of 2022 on OIV database (Gurgu & Fîntîneru, 2023). The Pietroasa produced annually 15.000 L/year Feteasca regala and 20.000 L-25.000 l/year Cabernet.
Our study focused on the behaviour of indigenous yeast in the vinification process at Pietroasa at the laboratory level.

MATERIALS AND METHODS

Samples of FR and CS grape varieties from the 2021 harvest (Figure 1) were collected from Pietroasa Research Station. The grapes were harvested in bins/trailers of 2 tons each and were transported to the winemaking line (Figure 2). After unloading in the reception bunker, the grapes reached the de-stemming machine and were crushed in order to obtain must.

Figure 1. Feteasca Regala (FR) and Cabernet Sauvignon (CS) grape varieties

Isolation and identification method of yeast strains

The grapes and must were subjected to yeast isolations. Both microscopic and macroscopic aspects were analyzed for the isolated yeasts.
Yeast pure colonies were isolated using the YPG (yeast, peptone hy soy and glucose) agarised media.
Yeast Peptone Glucose is a complete medium used for cultivation of a wide range of yeasts, including *Saccharomyces cerevisiae* (Dobrowolski, 1993; Feng et al., 2021).
The isolation was carried out using the classic methods:

- The technique of exhausting the loop on solid media-YPG/YPD (yeast extract, peptone, glucose, agar-agar) distributed in Petri dishes.

Isolated colonies were obtained from samples with high microbial density by the streak method;

- The technique of disseminating the

diluted sample on the surface of solid media (Begea et al., 2012; Cîmpeanu et al., 2010).
The technique involves going through two consecutive stages, 2-3 repetitions:

- dilution of the sample by the method of decimal dilutions;
- dilutions were made from each sample and were dispersed in Petri dishes on the specific culture media.

In order to obtain pure isolated colonies, the lawn depletion technique was used.
After seeding 0.1 ml/plate, we incubated the plate at 30^0C with a thermostat (Memmert GmbH + Co. (Schwabach, Germany) for 48-96 h.
After the incubation period, the colonies developed on the surface of the culture medium could be distinguished by their shape, size and color.
We isolated more than 5 pure colonies, which were then subjected to identification by molecular tests. MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight) technique was adopted for the identification of yeast strains.

Obtaining active dry yeast biomasses

The identified yeasts were used to obtain active yeast biomasses using a fermentation medium based on sterile diluted grape must (FR and CS) and a synthetic medium with glucose as the carbon source (Table 1).

The active yeast biomasses were then freeze-dried (data not shown) (Barbulescu et al., 2021).

Figure 2. Obtaining FR must for winemaking at Pietroasa winery

Obtaining must and wine from Feteasca regala (FR) grapes

The must was passed through a tube-in-tube heat exchanger, where the whole mass reached a temperature of 12-14^{0}C, and then went through a pneumatic press. After pressing, SO_2 was added to the must for protection and transported in a stainless steel vessel to be clarified with the help of deburring enzymes. At the end, the clear must was extracted from the upper part and sent to the vessels where the micro-samples were established for all the experimental variants. Subsequently selected yeasts and organic nutrients were then added to facilitate alcoholic fermentation. After fermentation (2-3 weeks) at ambient temperature of 17-19°C, the wines were conditioned and transferred into special 1.5 L vessels in 2 replications.

Obtaining rosé wine from Cabernet Sauvignon (CS) grapes

The CS obtained must was recirculated for 2 hours for an easy extraction and SO_2 was added for antioxidant protection. After maceration, the must was drained by gravity from the vessel and de-stemmed to be able to extract only the clear part. Finally, the clear must was sent to the vessels where the microsamples for all the experimental variants were established. Thus, the selected yeasts and the organic nutrients were added to facilitate the alcoholic fermentation followed the same procedure as for FR in the 1.5 L vessels Laboratory scale fermentation for winemaking was performed in 1.5 L bottles. Experimental wines were obtained based on the following protocol: 1.5 L must + dry yeast biomass + ET (Energyvin Thiols) and NO (Nutristart® ORG) nutrients (Table 2 and Figure 4).

Table 1. Yeast isolates used for obtaining active dry biomass

Sample code	Strain	Fermentation media used to obtain yeast biomass
F3A	*Saccharomyces* 1	FR must with 9.8% initial soluble dry matter, with yeast extract
F3B	*Saccharomyces* 2	CS must with 9.8% initial soluble dry matter, with yeast extract
F4A	*non-Saccharomyces* 2	CS must with 11.9% initial soluble dry matter, with yeast extract
F4B	*non-Saccharomyces* 2	CS must with 11.9% initial soluble dry matter, without yeast extract
F5B	*non-Saccharomyces* 1	FR must with 7.0% initial soluble dry matter, with yeast extract
F6A	*non-Saccharomyces* 3	Synthetic media with 7% glucose and yeast extract

Table 2. Samples of wine yeast biomass taken in vinification study

Sample code	Must variety	Nutreints	Name of the samples
F3A+F5B+ET	CS	Energyvin Thiols	*Saccharomyces* 1, Non-*Saccharomyces* 1
F3B+NO	CS	Nutristart Org	*Saccharomyces* 2
F4A+ET	CS	Energyvin Thiols	Non-*Saccharomyces* 2
F4B+NO	CS	Nutristart Org	Non-*Saccharomyces* 2
F3A+F5A+NO	FR	Nutristart Org	*Saccharomyces* 1, Non-*Saccharomyces* 1
F3A+NO	FR	Nutristart Org	*Saccharomyces* 1
F4A+ET	FR	Energyvin Thiols	Non-*Saccharomyces* 2
F5B+NO	FR	Nutristart Org	Non-*Saccharomyces* 1
F6A+ET	FR	Energyvin Thiols	Non-*Saccharomyces* 3

Analyses performed for FR and CS wines

Determination of alcohol concentration

The determination of the alcoholic strength by volume (% vol) of wine was performed by densimetry using hydrostatic balance, by the OIV-OENO 601B-2021 method. An oenological electronic distilling unit (Super D.E.E.) and a hydrostatic balance (Densi Alcomat) were used (Gibertini; Milano, Italy).

Determination of total sugar

The determination of the sugar content (g/L) in wine was performed by clarification with neutral lead acetate for red wine and zinc ferrocyanide (II) for white wine, followed by the determination of sugars using alkaline copper salt solution and titration with sodium thiosulfate solution, according to OIV-MA-AS311-01A method.

Determination of total acidity (expressed as tartaric acid)

The determination of the total wine acidity (g/L) was performed by titration with bromothymol blue as an indicator and comparison with an end-point color standard, using the OIV-MA-AS313-01: R2015 method.

RESULTS AND DISCUSSIONS

We isolated 5 yeasts of pure culture (Table 3 and Figure 3). The microscopic and macroscopic aspect is presented below:

The identified yeasts were 2 belonging to *Saccharomyces* and 3 non-*Saccharomyces*.

At the beginning of the vinification process, the sugar content was 19.5% (9.4% glucose and 10.5% fructose) for FR must, respectively, 22.1% (10.7% glucose and 11.4% fructose) for CS must.

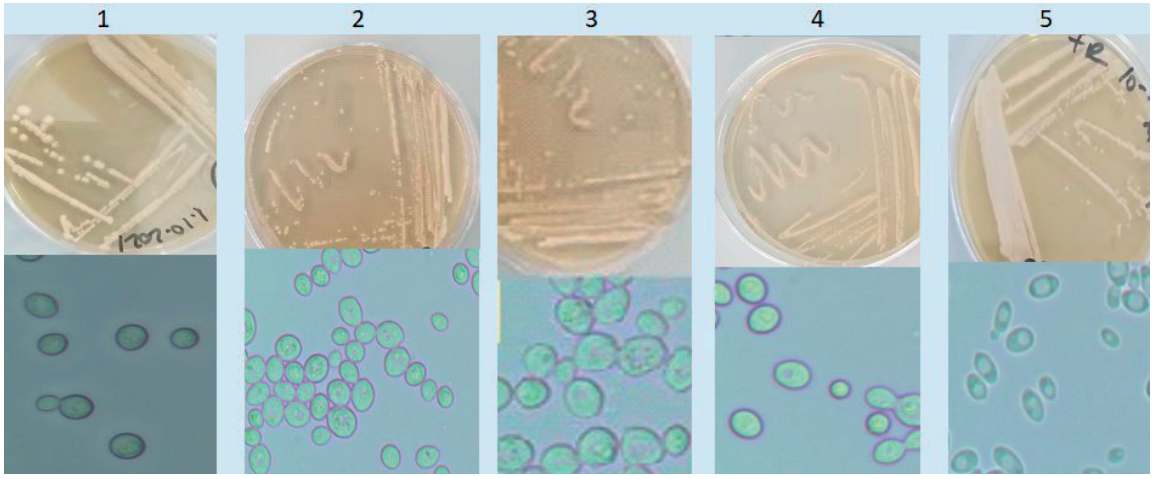

Figure 3. Macroscopic (1 - *Saccharomyces* 1; 2 - *Saccharomyces* 2; 3 - *non-Saccharomyces* 1*; 4 - non-Saccharomyces* 2*; and 5- non-Saccharomyces* 3) and microscopic (1 - *Saccharomyces* 1; 2 - *Saccharomyces* 2; 3 - *non-Saccharomyces 1; 4 - non-Saccharomyces 2 and 5 - non-Saccharomyces* 3) aspects of yeast cells

Table 3. Macroscopic and microscopic aspects of the isolated yeasts

Sample code	Material	Variety	Microscopic aspect	Macroscopic aspect
Saccharomyces 1	Grapes	FR	Cells elongated, cylindrical	Round creamy white colonies
Saccharomyces 2	Must	CS	Oval cells	Oval cell colonies
non-Saccharomyces 1	Must	FR	Round cells	Round colonies, light cream-white in color, uniform appearance, pure culture, slightly mucilaginous appearance
non-Saccharomyces 2	Grapes	CS	Round cells, budding	Creamy white colonies
non-Saccharomyces 3	Must	FR	Apiculate cells, budding, cells separated from the mother cell	White, round colonies

Dry active yeast is a crucial component in winemaking, as it plays a significant role in the fermentation process. This measurement is essential because it determines the yeast's ability to effectively carry out fermentation and contribute to the wine's desired characteristics. It is typically measured in terms of the number of viable yeast cells per gram of yeast, expressed as colony-forming units (UFC) per gram.

From the determinations performed on the analyzed samples, it was observed that the viability for the dry active yeast was between 1.08×10^6 UFC/g and 2.1×10^8 UFC/g, which is considered optimal for winemaking.

Table 4. Recorded results for wine samples from the 2021 harvest

Trial	Sample code	Grape variety	Alcohol concentration (% vol)	Acidity (g/l tartaric acid)	Reducing sugar (g/L)
1	F3A-NO -FR	Feteasca regala	12.61	4.35	3.63
2	F3B+NO -CS	Cabernet Sauvignon	16.21	7.35	6.40
3	F4B+NO - CS	Cabernet Sauvignon	16.44	7.28	6.40
4	F4A+ET CS	Cabernet Sauvignon	16.19	7.28	6.80
5	F5B-NO-FR	Feteasca regala	12.33	4.58	4.38
6	F6A-ET-FR	Feteasca regala	11.13	3.98	5.33
7	F4A-ET-FR	Feteasca regala	12.64	4.58	4.38
8	F3B+F5B+NO - CS	Cabernet Sauvignon	16.17	7.65	8.00
9	F3A+F5A+NO -FR	Feteasca regala	13.70	4.80	3.20

Figure 4. Wine samples by Feteasca regala and Cabernet Sauvignon obtained at laboratory level vessel by 1.5 L

According to Table 4 the best results were obtained for the FR grape must fermented with F3A+F5A+NO - FR. The wine obtained had the following physical-chemical characteristics: 13.7% vol alcohol concentration; 4.8 g/L total acidity (tartaric acid); 3.2 g/L reducing sugar. The FR must's initial sugar content was 19.45% (9.4% glucose and 10.45% fructose) (Table 4). Therefore, for these experiments the best transformation in alcohol with a good acidity of wine was noticed. Călugăr et al. (2019) demonstrated that the alcoholic strength has varied from a maximum of 12.62% in the control sample and a minimum of 12.49% for the sample aged for 90 days in oak barrel for Feteasca regala variety. Total acidity (titratable acidity) is defined as the total substances with acid reaction present in wine, which can be titrated with an alkaline solution in the presence of an indicator. Total acidity values obtained are specific vine variety investigated, and also the values of aged samples are in the normal rage. Antoce and Cojocaru (2015) studied the obaining of wines of Feteasca regala variety by inoculation and co-inoculation with several commercially available wine yeasts. and they found out that the grapes had high sugar content at harvest. The wines produced by fermentation with the selected yeasts had more than 15% v/v alcohol concentration, which is unusual for the Feteasca regala variety (Antoce & Cojocaru, 2021). In addition, for the F4A-ET variants in both CS and FR, it was observed that the results for the FR wine are specific for a more balanced wine in terms of acidity and of alcohol concentration in comparison with the CS wine sample obtained with the same yeast biomass. Moreover, a higher concentration of alcohol and acidity was observed for F3B+NO - CS wine, in comparison to the F3A-NO-FR wine.

CONCLUSIONS

Different isolated yeasts were tested, both at the laboratory and micropilot level, to obtain active yeast biomass.

Since we aim to preserve the typicality of the wine, we specifically aimed to carry out the fermentations in a natural culture media based on grape must. Given that the must can be found only in the winemaking campaigns, we also achieved the production of yeast biomass on synthetic must with glucose and sucrose as carbon sources. In conclusion, the most promising results for winemaking were obtained from combining different yeast strains: F3A+F5A+NO: *Saccharomyces* 1, non-*Saccharomyces* 1, and the complex nutrient Nutristart Org.

In the following studies, we plan to detect the distinct flavours of the wines obtained based on the technology developed and tested in the present study, through the influence of different yeast cultures, with the main aim to preserve the concept of "terroir" at Pietroasa winery by promoting local, autochthone wines obtained with native yeasts.

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REFERENCES

Alonso-del-Real, J., Contreras-Ruiz, A., Castiglioni, G. L., Barrio, E. and Querol, A. (2017). The Use of Mixed Populations of *Saccharomyces cerevisiae and S. kudriavzevii* to Reduce Ethanol Content in Wine: Limited Aeration, Inoculum Proportions, and Sequential Inoculation. *Front. Microbiol., 8*: 2087. doi: 10.3389/fmicb.2017.02087.

Antoce, A. O., Cojocaru, G. A. (2015). Effects of Blending and Co-inoculation on the Aromatic Profile of Wines, *Rev. Chim. (Bucharest), 66*, No. 10.

Antoce, A. O., Cojocaru, G. A. (2021). Evaluation by Flash GC Electronic Nose of the Effect of Combinations of Yeasts and Nutrients on the Aromatic Profiles of Feteasca Regala Wines after Two Years of Storage. *Fermentation, 7*, 223. https://doi.org/10.3390/ fermentation7040223.

Barbulescu, I. D., Ghica, M. V., Begea, M., Albu Kaya, M. G., Teodorescu, R. I., Popa, L., Mărculescu, S. I., Cîrîc, A. I., Dumitrache, C., Lupuliasa, D. et al. (2021). Optimization of the Fermentation onditions for Brewing Yeast Biomass Production Using the Response Surface Methodology and Taguchi Technique. *Agriculture, 11*, 1237. https://doi.org/10.3390/agriculture11121237.

Begea, M., Sirbu, A., Kourkoutas, I., Dima, R. (2012). Single cell protein production of *Candida strains* in culture media based on vegetal oils. *Romanian Biotechnological Letters, Vol. 17*(6), 7776-7786.

Bougreau, M., Ascencio, K., Bugarel, M., Nightingale, K., Loneragan, G. (2019). Yeast species isolated from Texas High Plains vineyards and dynamics during spontaneous fermentations of Tempranillo grapes. PLoS ONE 14(5): e0216246. https://doi.org/10.1371/journal.pone.0216246.

Brežná, B., Ženišová, K., Chovanová, K., Chebeňová, K´., Kraková Tomáš, V., Kuchta, L., Domenico Pangallo, D. (2010). Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. *Anton Leeuw, 98*: 519-29. 10.1007/s10482-010-9469-6.

Călugăr, A., Pop, T. I., Coldea, T. E., Mureșan, I., Gal, E., Manolache, M. (2019). Eonological parameters of Fetească regală wines subjected to aging with oak chips of and barrel during three periods of time. *Journal of Horticulture, Forestry and Biotechnology Vol. 23*(1), 78- 83. www.journal-hfb.usab-tm.ro.

Cioch-Skoneczny, M., Satora, P., Skotniczny, M. and Skoneczny, S. (2018). Quantitative and qualitative composition of yeast microbiota in spontaneously fermented grape musts obtained from cool climate grape varieties 'Rondo' and 'Regent'. *FEMS Yeast Research, 18*, foy089, doi: 10.1093/femsyr/foy089.

Cîmpeanu, C., Câmpeanu, G., Begea, M., Vlădescu, M., Cornea, P. (2010). Bioethanol production by new thermotolerant Romanian yeast strains. *Romanian Biotechnological Letters, Vol. 15*(3), 5310-5316.

Colombo, F., Di Lorenzo, C., Regazzoni, L., Fumagalli, M., Sangiovanni, E., de Sousa, L. P., Bavaresco, L., Tomasi, D., Bosso, A., Aldini, G. et al. (2019). Phenolic profiles and anti-Inflammatory activities of sixteen table grape (*Vitis vinifera* L.) varieties. *Food Funct., 10*, 1797-1807. Doi https://doi.org/10.1039/C8FO02175A.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I. et al. (2012). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae. Food Microbiol., 28*(5): 873–82. https://doi.org/10.1016/j.fm.2010.12.001 PMID: 21569929.

Dobrowolski, P. (1993). Short protocols in molecular biology. A compendium of methods from "current protocols in molecular biology. Edited by Frederick, M. Ausubel et al., John Wiley & Sons, 1992, ISBN 0-471-57735-9. Acta Biotechnologica, Microbial and Chemical Analysis of Non-*Saccharomyces* 13, 88.

Drumonde-Neves, J., Franco-Duarte, R., Lima, T. et al. (2017). Association between grape yeast communities and the vineyard ecosystems. PLoS One 2017; 13:1-17.

Feng, C. T., Du, X. and Wee, J. (2021). Yeasts from Chambourcin Hybrid Grapes for Potential Use in Winemaking. *Fermentation, 7*, 15. https://doi.org/10.3390/fermentation7010015, https://www.mdpi.com/journal/fermentation.

Gurgu, A. E., Fîntîneru, G. (2023). The potential of romanian wineries in wine tourism development. Scientific *Papers Series Management, Economic Engineering in Agriculture and Rural Development Vol. 23*, Issue 2, PRINT ISSN 2284-7995, E-ISSN 2285-3952.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.; 33*(2): 271–81. https://doi.org/10.1016/j.fm.2012.10.004 PMID: 23200661.

Kapsopoulou, K., Mourtzini, A., Anthoulas, M., Nerantzis, E. (2007). Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans and Saccharomyces cerevisiae*. *World J Microb Biot., 23*(5): 735–9. Doi 10.1007/s11274-006-9283-5.

Ramírez, M. and Velázquez, R., (2018). The Yeast *Torulaspora delbrueckii*: An Interesting But Difficult-To-Use Tool for Winemaking. *Fermentation, 4*, 94. doi:10.3390/fermentation4040094.

Unusan, N. (2020). Proanthocyanidins in grape seeds: An updated review of their health benefits and potential uses in the food industry. *J. Funct. Foods, 67*, 103861.

Zhou, D. -D., Li, J., Xiong, R. -G., Saimaiti, A., Huang, S. -Y., Wu, S. -X., Yang, Z. -J., Shang, A., Zhao, C. -N., Gan, R. -Y. et al. (2012). Bioactive Compounds, Health Benefits and Food Applications of Grape. *Foods, 11*, 2755. https://doi.org/10.3390/foods11182755.

Website: https://www.pietroasaveche.ro/vinuri-2.

***OIV-OENO 601B-2021. Revision of the method OIV-MA-AS312-01A: Alcoholic strength by volume at 20°C.

***OIV-MA-AS311-01A. Reducing substances, Resolution OIV/Oeno 377/2009.

***OIV-MA-AS313-01. Total acidity, revised by OIV/OENO 551-2015.