

Proceeding Paper

Molecular and Phenotypic Diversity of Indigenous Oenological Strains of *Saccharomyces cerevisiae* Isolated in Greece †

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Abstract: During 3 years, we explored the biodiversity of the indigenous yeast flora in five Greek wine regions by collecting five varietal grape samples, conventionally and biologically cultured. Spontaneous wine fermentations were carried out by the native microbiota of the grape juice, without the inoculation of selected industrially produced yeast. The indigenous yeast flora, isolated at three phases of these fermentations, was purified and characterized using different oenological and technological criteria. The pre-selected *Saccharomyces cerevisiae* strains, with the most promising oenological characteristics, were evaluated in microvinifications of Malagousia must and the quality of the produced wines was subjected to a sensorial descriptive analysis.

Keywords: indigenous *Saccharomyces cerevisiae*; yeast strain selection; alcoholic fermentation



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1. Introduction

Greece is the 17th wine-growing country in the world, representing 1.3% of European wine production with a small average vineyard ownership (3.95 acres) [1]. There are more than 300 Greek indigenous varieties, cultivated single or in combination with international varieties in 9 different wine-growing regions (mainland and islands), in soils that vary strongly, with altitudes from 0 to over 1000 m. Greece has 33 Protected Designation of Origin (PDO) zones and more than 100 Protected Geographical Indication (PGI) zones. In a highly competitive international environment, Greek agriculture has high production costs and competition from already established wine-producing countries. There is great interest in creating typical products in relation to geographical names. Value creation relies on the wine's ability to satisfy consumers in the long run. Sensory complexity is a critical indicator of quality [2] and the concept of wine complexity is increasingly of interest to scientists, winemakers, and wine lovers. Yeasts allow the complete and rapid conversion of sugars into ethanol and CO₂, and their intervention also contributes to the taste of wines [3]. A large number of microbial species, mainly yeasts, follow and replace each other in grape must during winemaking [4]. Only two species can complete the fermentation, metabolizing the total concentration of sugars: *S. cerevisiae* and, in to a lesser degree, *S. uvarum*. There are also other genera of yeast in winemaking, present in different stages, pre-fermentative or at the beginning of the fermentation. They are known as non-*Saccharomyces* (NS) and have long been considered undesirable. Scientific research has shown that many of them have a technological interest. In the context of the production of unique products, there is a lot of discussion about the "indigenous" yeasts, which are naturally present in grapes and must. These spontaneous fermentations could lead to problematic fermentations. For the best fermentation control, about 80% of them

are conducted by commercial yeast inoculations. *S. cerevisiae* remains the main commercial yeast marketed by oenological companies, in the form of LSA (Active Dry Yeast).

2. Materials and Methods

Grapes samples were collected in their technological maturity in three different growing seasons (2018–2020). Malagousia (mostly), Assyrtiko, Vidiano, Moschofilero, and Agiorgitiko were collected in five wine-producing PGI zones: Drama, Pangeon, Chalkidiki, Thessaloniki, and Atalanti Valley. The mode of culture was either conventional or biological. Directly fresh grapes or defrosted were destemmed and crushed by hand. The grape mass obtained, 25 L for each batch, was fermented spontaneously at 25 °C. Diammonium phosphate (20 g hL⁻¹) was added to the grape must. All the spontaneous fermentations took place in 30 L stainless steel thermo regulated tanks, exact copies of professional winemaking tanks (Figure 1). Isolation, purification, and conservation of cultures: the indigenous yeast flora was isolated a) from grapes and b) during three phases of the fermentations (beginning, middle (6 Baume), and end (<1 Baume)). Successive dilutions of fermenting musts were placed in culture and incubated for 5 days at 25 °C [5–7]. Aliquots of several decimal dilutions in 0.1% peptone water were spread onto YPG Nutrient Agar that had been treated with streptomycin sulfate (250 mg L⁻¹). A series of coatings were performed by the method of linear coating on the agar culture plates (YPG + streptomycin sulfate) method to obtain clean cultures. The operation was renewed by randomly taking an isolated colony each time. Microbiological identification: Yeast isolates were identified by phenotypic criteria [8]. Macroscopic and microscopic observations of isolation yeasts were also performed in YPG and Chromagar (Figure 2) [9,10]. The identification system ID 32C was used for the carbon assimilation tests. ID 32 C is a standardized system for the identification of yeasts, which uses 32 miniaturized assimilation tests and a database. Molecular identification: DNA extraction was performed (Genomic DNA from tissue-Macherey-Nagel-01/2017, Rev.17). Quantification and testing of DNA purity was conducted [11]. Random amplification of polymorphic DNA (RAPD-PCR), PCR fingerprinting, and interdelta PCR typing [12], followed by detection of PCR products (electrophoresis). Enzymatic profile: An API ZYM system was used for enzymic profiling in order to evaluate the strains' potential, because of the involvement of certain enzymes in the vinification process [13]. Oenological criteria: The isolated yeasts were tested according to several criteria: production of hydrogen sulphide [14,15], flocculation properties [16], fermentation rate [17], ethanol tolerance, osmotolerance, high-temperature growth [18], malic and acetic acid production [19], and enzymatic activities [19,20]. Fermentations with selected yeast strains: Eight strains of *S. cerevisiae* selected for their oenological criteria were used for the inoculation (1%) of must obtained from Malagousia grapes. Fermentations were carried out at the Laboratory of Marketing and New Products Development, Department of Agricultural Biotechnology and Oenology, IHU. A total of 25 L of Malagousia grape musts were fermented in 30 L stainless steel thermo regulated tanks, having the following chemical characteristics: sugars, 209 g L⁻¹; pH 3.55; titrable acidity, 6.1 g L⁻¹ tartaric acid; and assimilable nitrogen, 80 mg L⁻¹. Musts were supplemented with 30 ppm total of SO₂. Nutrient additions were performed before the inoculation (organic nitrogen, 40 g hL⁻¹) and after 150 g L⁻¹ sugar consumption (organic and inorganic nitrogen, 40 g hL⁻¹). Pre-cultures of yeast strains were propagated in YPD agar at 26 °C. Cells were collected and re-suspended in Ringer's solution. Each strain was inoculated at approximately 100 cells mL⁻¹. Fermentations were conducted at 18 °C and the progress was monitored by density measurements. The quality of the produced wines was evaluated and subjected to a sensorial analysis [21]. **Typical chemical analysis:** The determination of chemical parameters on the must and wines was performed: reducing sugars, total and volatile acidity, pH, malic and lactic acid, and free and total sulfur dioxide [22]. Assimilable nitrogen was assayed using the formol method [23]. **Sensory analysis:** Sensory evaluation of the aroma (fruity and floral) and flavor (fruity, floral, sour, astringency, body, and after taste) of the wines fermented by the selected yeasts was performed by a panel of 10 judges/experts. Intensity ratings were

scored on scale from 1 (not perceivable) to 5 (very strong). **Statistical analysis:** Statistical data processing was applied to the sensory analysis, performed using Minitab Statistical Software.

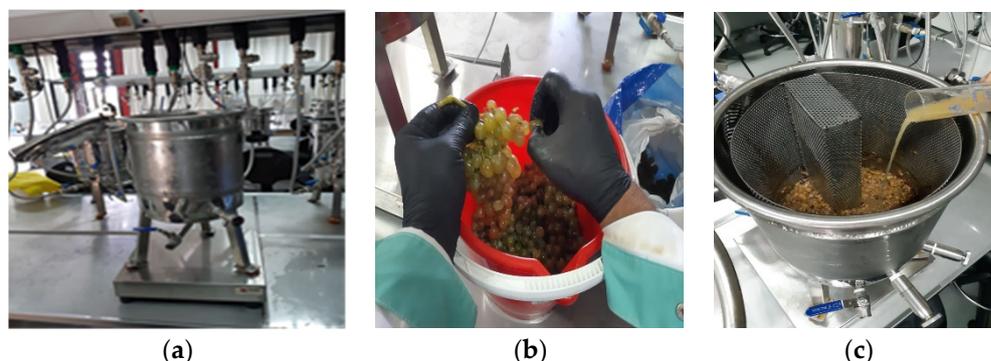


Figure 1. (a) Stainless steel fermentation tanks; (b) destemming of the grapes; and (c) initiation of spontaneous alcoholic fermentation.



Figure 2. *Saccharomyces cerevisiae* in a Chromagar medium.

3. Results and Discussion

Phenotypic typing and characterization of *S. cerevisiae* isolates of the 76 isolates studied showed that 18 were identified as *S. cerevisiae* (Figure 3). *S. cerevisiae* were mainly derived from integrated grapes. They were found in grapes of the same variety (Malagousia) of the same sampling vineyard and repeated for 3 consecutive years, while in another PGI zone (Drama) they were isolated year after year, similarly at the same variety and vineyard each year. The same *S. cerevisiae* strain was identified in the wines produced from the grapes of the first case at the end of the fermentation. Therefore, in integrated grapes of a given area (Pangeon), the same strain was isolated in the grapes for every year of the process, before and at the end of the fermentation. In organic grapes, *S. cerevisiae* had a lower incidence. So, they were identified in a single case, in the middle of the fermentation, and in the same variety and area (Thessaloniki) for 2 consecutive years. Various species that were used to classify the genus *Saccharomyces*, but were eventually considered synonymous with *S. cerevisiae*, are named by many researchers as breeds or normal varieties [24] (Table 1). In order to distinguish various *S. cerevisiae* strains, $\delta 1$ – $\delta 2$ and $\delta 12$ – $\delta 2$ primers amplifying inter-delta sequences were employed [12]. **Oenological properties:** The results of the flocculation tests showed that most of the strains (89%) remained in suspension after 10 min [25,26]. The isolated strains had zero-to-medium ability of hydrogen sulphide production and zero malic and acetic acid production. All the strains had glucosidase, but low or no beta glucosidase activity. Regarding their enzymatic profile, α -fucosidase, N-acetyl β -glucosaminidase, esterase, esterase/lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, aminopeptidase, phosphohydrolase, and galactosidase were detectable. Although each strain had a unique enzyme pattern, the mean enzyme activity was esterase, lipase, and esterase/lipase with slightly higher activity. The action of N-acetyl β -glucosaminidase shows maximum enzymatic activity, 40 nmoles, in almost all strains except from one. Zero activity is shown by β -galactosidase, occurring only in one strain with very low activity, 5 nmoles. Alkaline phosphatase,

α -chymotrypsin, and α -glucosidase were not detected in any of the strains. **Alcoholic fermentations:** Similar fermentation kinetics were observed in Sc2 and Sc6, Sc1 and Sc4, and Sc3 and Sc7. Higher fermentation rates and faster completion of the process were observed in Sc1 and Sc4. Two of the eight strains (Sc2, Sc4) did not metabolize the total initial sugars. Two strains gave wines with high volatile acidity. The duration of the fermentation varied from 9 to 17 days (Figure 4). At the end of the fermentations, a 750 mL centrifuged sample of each tank was taken for chemical analysis. In all cases, the pH values (3.56–3.89), alcoholic degree (11.75–12.95), volatile acidity (0.35–1.60 g acetic acid L⁻¹), and total acidity (5.0–6.9 g tartaric acid l⁻¹) ranged at usual levels [18,21]. **Sensory analysis:** Two months after fermentation, the lies were discarded and 3 bottles of each tank were prepared and tasted by a panel of 10 experts. Regarding the aromas, the conclusion for the overall assessment coincide with the individual assessments. From the average of the four indicators related to the aroma, some samples can be distinguished (those with the highest average values). Among these, one has an extremely low variability (standard deviation), which means that all its features are consistently high. The same sample gathered the best performance in the parameter “overall aroma rating”. This is important because it demonstrates the objectivity and effectiveness of the grading method.

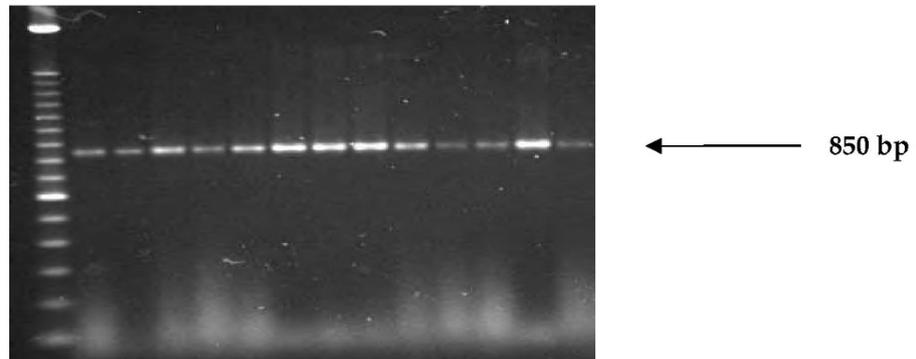


Figure 3. Agarose gel analysis of the PCR products.

Table 1. Different fermentative responses of indigenous wine *Saccharomyces cerevisiae*.

Fermentative Type	Isolate Identity *	Glu	Suc	Mal	Raf
I	MBm4	+	+	+	–
II	MCE2	+	+	+	+
III	Mci10	–	+	A	+
IV	Others	+	+	+	A

Glu, Glucose; Suc, sucrose; Mal, maltose; Raf, raffinose; +, fermentation positive; A, assimilation positive; –, fermentation and assimilation negative. * Capital letters indicate grape and type of viticulture, and small letters indicate fermentation stage: MCI, Malagousia must, Conventional culture, and initial stage. Arabic numbers represent the isolate number.

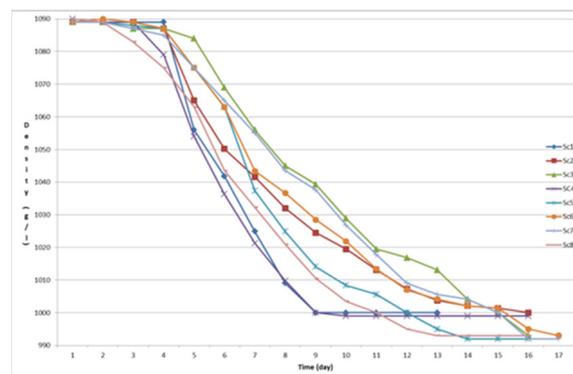


Figure 4. Cont.

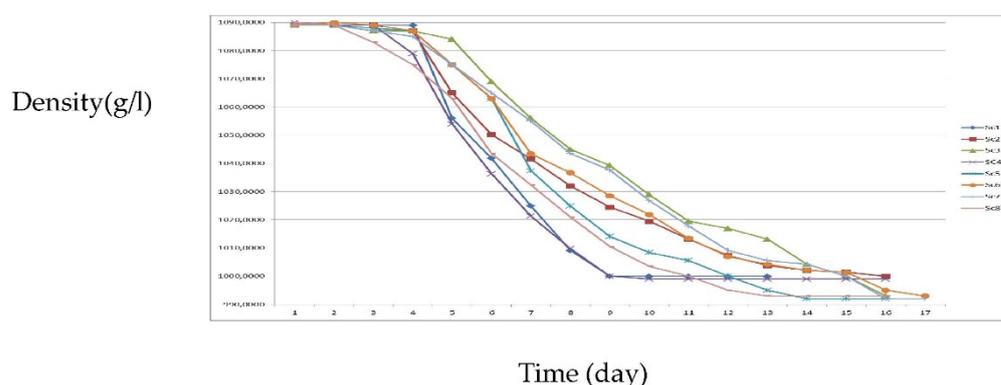


Figure 4. Kinetics of pilot-scale fermentations of inoculated grape must with indigenous *S. cerevisiae*.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/engproc2021009007/s1>: Figure S1: Fermentation tanks; Figure S2: *S. cerevisiae* in Chromagar; Figure S3: Electrophoresis of the PCR products; Figure S4: Kinetics of pilot-scale fermentations; Table S1: Different fermentative responses of *S. cerevisiae* strains.

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References

- OIV. 2019 *Statistical Report on World Vitiviniculture*; OIV: Paris, France, 2018.
- Dubourdieu, D. La valeur du vin entre nature et culture, réflexions sur le goût mondial et la typicité des vins. In Proceedings of the OENO 2011, 9e Symposium International d’Œnologie de Bordeaux, Paris, France, 15 June 2012; pp. 3–6.
- Swiegers, J.H.; Bartowsky, E.J.; Henschke, P.A.; Pretorius, I.S. Yeast and bacterial modulation of wine aroma and flavor. *Aust. J. Grape Wine Res.* **2005**, *11*, 139–173. [[CrossRef](#)]
- Walker, G.M.; Stewart, G.G. *Saccharomyces cerevisiae* in the Production of Fermented Beverages. *Beverages* **2016**, *2*, 30. [[CrossRef](#)]
- Berber, N.; Aissaoui, R.; Ali Bekada, A.M.; Coarer, M. Isolation and Molecular Identification (PCR-Delta and PCR-RFLP-ITS) of the yeast from Black muscat grape cultivated in El malah (Algeria). *Adv. Environ. Biol.* **2016**, *10*, 55–61.
- Renouf, V.; Claisse, O.; Lonvaud-Funel, A. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Aust. J. Grape Wine Res.* **2005**, *11*, 316–327. [[CrossRef](#)]
- Résolution OIV-OENO 576A-2017. *Monographie Sur Les Levures Saccharomyces Sofia 2 Juin 2017*; OIV: Paris, France, 2017.
- Barnett, J.; Payne, R.; Yarrow, D. *Yeasts: Characteristics and Identification*; Cambridge University Press: Cambridge, UK, 2000.
- Ainscough, S.; Kibbler, C. An evaluation of the cost-effectiveness of using Chromagar for yeast identification in a routine microbiology laboratory. *J. Med. Microbiol.* **1998**, *47*, 623–628. [[CrossRef](#)]
- Koehler, A.P.; Chu, K.C.; Houang, E.T.S.; Cheng, A.F.B. Simple, Reliable, and Cost-Effective Yeast Identification Scheme for the Clinical Laboratory. *J. Clin. Microbiol.* **1999**, *37*, 422–426. [[CrossRef](#)] [[PubMed](#)]
- Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; University of Texas South Western Medical Center: Dallas, TX, USA, 1989.
- Legras, J.-L.; Karst, F. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. *FEMS Microbiol. Lett.* **2003**, *221*, 249–255. [[CrossRef](#)]
- Canal-Llauberes, R.M. *Effect of Pre-Fermentative Strategies on the Composition of Prieto Picudo (Vitis vinifera) Red Wines*, *Wine Microbiology and Biotechnology*; Harwood Academic Publishers: Reading, UK, 1993; Volume 3.

14. Giudici, P.; Kunkee, R.E. The Effect of Nitrogen Deficiency and Sulfur-Containing Amino Acids on the Reduction of Sulfate to Hydrogen Sulfide by Wine Yeasts. *Am. J. Enol. Vitic.* **1994**, *45*, 107–112.
15. Zambonelli, C. *Microbiologia e Biotecnologia dei Vini*; Edagricole-New Business Media: Bologna, Italy, 1998; p. 300.
16. Bony, M.; Bidart, F.; Camarasa, C.; Ansanay, V.; Dulau, L.; Barre, P.; Dequin, S. Metabolic analysis of *S. cerevisiae* strains engineered for malolactic fermentation. *FEBS Lett.* **1997**, *410*, 452–456. [[CrossRef](#)]
17. Pérez-Coello, M.S.; Briones Pérez, A.I.; Ubeda Iranzo, J.F.; Martín Alvarez, P.J. Characteristics of wines fermented with different *Saccharomyces cerevisiae* strains isolated from the La Mancha region. *Food Microbiol. Dec.* **1999**, *16*, 563–573. [[CrossRef](#)]
18. Regodón, J.A.; Pérez, F.; Valdés, M.E.; De Miguel, C.; Ramírez, M. A simple and effective approach for selection of wine yeast strains. *Food Microbiol.* **1997**, *14*, 247–254. [[CrossRef](#)]
19. Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, *28*, 873–882. [[CrossRef](#)] [[PubMed](#)]
20. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
21. Nikolaou, E.; Soufleros, E.H.; Bouloumpasi, E.; Tzanetakis, N. Selection of indigenous *Saccharomyces cerevisiae* strains according to their oenological characteristics and vinification results. *Food Microbiol.* **2006**, *23*, 205–211. [[CrossRef](#)] [[PubMed](#)]
22. *Compendium of International Methods of Wine and Must Analysis OIV, Edition 2021*; OIV: Paris, France, 2020.
23. Gump, B.H.; Zoecklein, B.W.; Fugelsang, K.C.; Spencer, J.F.T.; De Spencer, A.L.R. Prediction of Prefermentation Nutritional Status of Grape Juice: The Formol Method. *Food Microbiol. Protoc.* **2003**, *14*, 283–296.
24. Ribéreau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. *Traite D'œnologie, 2. Chimie du vin, Stabilisation et Traitements*; Dunod: Paris, France, 2000.
25. Ortiz, M.J.; Barrajon, N.; Baffi, M.A.; Arévalo-Villena, M.; Briones, A. Spontaneous must fermentation: Identification and bio-technological properties of wine yeasts. *LWT* **2013**, *50*, 371–377. [[CrossRef](#)]
26. Suranská, H.; Vránová, D.; Omelková, J. Isolation, identification and characterization of regional indigenous *Saccharomyces cerevisiae* strains. *BJM* **2016**, *47*, 181–190. [[CrossRef](#)] [[PubMed](#)]