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ISOLATING WINE YEASTS THAT ARE SPECIFIC TO THE APOLD REGION AND IDENTIFYING THEM THROUGH RFLP GENETIC METHODS

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ABSTRACT. The present study aims at isolating, identifying and selecting autochthonous wine yeast strains with a view to establish a crop bank specific to the Apold area. 569 wine yeast strains were isolated during the alcoholic fermentation of must from the Apold area, 458 were identified through cultural methods and with the help of the API 20 C AUX test (Biomeriux, France). Six yeast strains (A87, A169, A296, A314, A132 and A413) were genetically identified through the PCR-ITS RFLP method of the 5.8S-ITS segment; the resulting four strains were *Saccharomyces cerevisiae* - A87, A169, A296, A314 - and two *Saccharomyces bayanus* strains - A132 și A413. The strains we identified constitute a base for the multiplication of indigenous species with a view to obtain authentic wines that are typical to their area of origin.

1. INTRODUCTION

Wine yeasts isolated from the epiphytic microflora of a wine region represent an ever greater interest for producers who want to obtain distinct wines specific to the area. These specific traits can be imposed by selecting indigenous wine yeast cultures of a higher biotechnological character, thus eliminating the aromatic leveling of wines by using commercial yeasts. Microorganisms, especially yeasts, are mostly found on grapes and on grapevines; they get here due to insects, air currents, precipitations and also dust particles. During the grape processing stages, yeasts get into the must, where they play a part in the alcoholic fermentation processes [1, 2]. Grape must contains a great variety of microorganisms, such as bacteria, yeasts, molds, but which, depending on physical and chemical factors, can survive or not. Microorganisms that were frequently identified in musts fall into the genera *Brettanomyces, Pichia, Torulopsis, Kloeckera, Saccharomyces, Candida, Penicillium, Aspergillus, Absidia, Botrytis* [3, 4]. *Saccharomyces* yeasts confer specific regional characters to wines and can enhance aromas in grapes, respectively in must, leading to wine typicality. Isolating these yeasts in a pure culture is a decisive factor, as their properties lead to obtaining wines that are specific, natural, having authentic and personalized aromas [5, 4]. Resulting wines will bear the aromatic mark of variety, of the technological procedures implemented, but also of the area of origin [6, 7]. Genetic identification is a priority, given the diversity of yeasts in must, as present methods are ever more simple and exact [8].

2. MATERIALS AND METHODS

-Grape must from the Apold wine region, harvest of 2014, undergoing alcoholic fermentation at 20^{o}C for 10 days in a Fermentas laboratory fermentator, with a capacity of 2 L, equipped with sensors for temperature, pH, CO_2, O_2, a data acquisition plate, thermostat for temperature adjustment, water recirculation pump through the double sheathed fermentation vessel.
-Culture medium: malt-agar must produced by Scharlau, Spain, for the multiplication of yeats API 20 C AUX (Biomeriux, France) tests which quickly determine the genus of yeasts in a biomass, as they are based on the property of yeasts to assimilate sugars; they are identified through a six figure numerical code. The method involves the introduction of fresh yeast in a test tube containing 2 ml of API NaCl 0,85% environment. We take 100 µL of this suspension and inoculate it in 7 ml API C

environemt. According to its recipe, the API C environment contains: ammonium sulfate 5 g, mono-potassium phosphate 0.31 g, dipotassium hydrogen phosphate 0.45 g, disodium diphosphate 0.92 g, sodium chloride 0.1 g, calcium chloride 0.05 g, magnesium sulphate 0.2 g, L-histidine 0.05 g, gelling agent 0.5 g, vitamin solution 1 ml, dietary-element solution 10 ml, demineralised water 1000 ml. Using a pipette, the cells are filled with the final suspension and are left to incubate for 48-72 hours at a temperature of 29° C. The positive (colored) cells are noted with a plus, then the values obtained are added up for each group of substrate; the result is a code made up of 6 figures, which is introduced in the related API web catalogue, which indicates the genus/species identified.

In order to select wine yeasts, we take succesive samples for 6 days; sowing is done in malt must in Petri plates. Among the samples analysed from the point of view of culture and morphology, we selected those that presented similarities with the *Saccharomyces* genus, as they are undergoing API 20 C AUX identification tests according to the above-described method.
Pre-identified yeast strains of the *Saccharomyces* genus were genetically tested through the method described by [9] after [10]. They were identified through the PCR-ITS RFLP analysis of the 5.8S-ITS segment.
As primers, we used: ITS 1 (5'- TCCGTAGGTGAACCTGCGG – 3') and ITS 4 (5'- TCCTCCGCTTATTGATATGC – 3'); amplification took place in 40 cycles at a temperature of 95°C for 30 seconds, then at 52°C for 1 minute and finally at 52°C for 1 minute, in a MultiGene OptiMax Thermal Cycler. Enzymatic digestion was done using 3 enzymes: *Cfo*I, *Hae*III and *Hinf*I. DNA fragment vizualization was done in 2% agarose gel in UV in the presence of ethidium bromide. The dimension of PCR products was estimated through a comparison with a standard DNA length (GeneRuler 100bp Gibco-BRL).

3. RESULTS AND DISCUSSIONS

Grape must alcoholic fermentation is an anaerobic process involving a series of reactions turning sugars into alcohol, CO_2 and by-products under the influence of wine yeasts. Wine yeasts play an extremely important role in these processes, as their amount and quality contribute to the formation of the wine bouquet as they can potentiate the aromas in the must. Monitoring this process led to the diagrams in figure 1, which shows that pH evolves downward compared to time; the values obtained oscilate between 6.5 and peaks of 7.8, finally settling at 6.4.

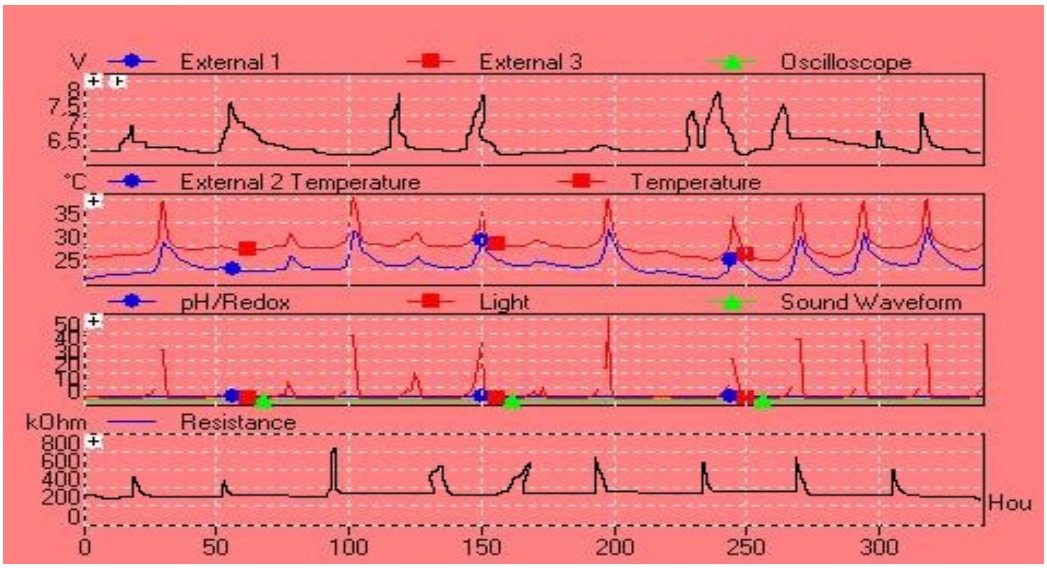

Figure 1. Graph showing the alcoholic fermentation of must made of grapes from Apold, production of 2014

During grape must alcoholic fermentation, a series of microorganisms are exponentially amplified; the biggest proportion is that of microorganisms in the *Saccharomyces* genus. In this experiment, we isolated 569 yeast strands, of which 458 were identified using API 20 C AUX tests according to the examples in figures 2 and 3; the results confirmed that these yeasts fell into the *Saccharomyces* genus.

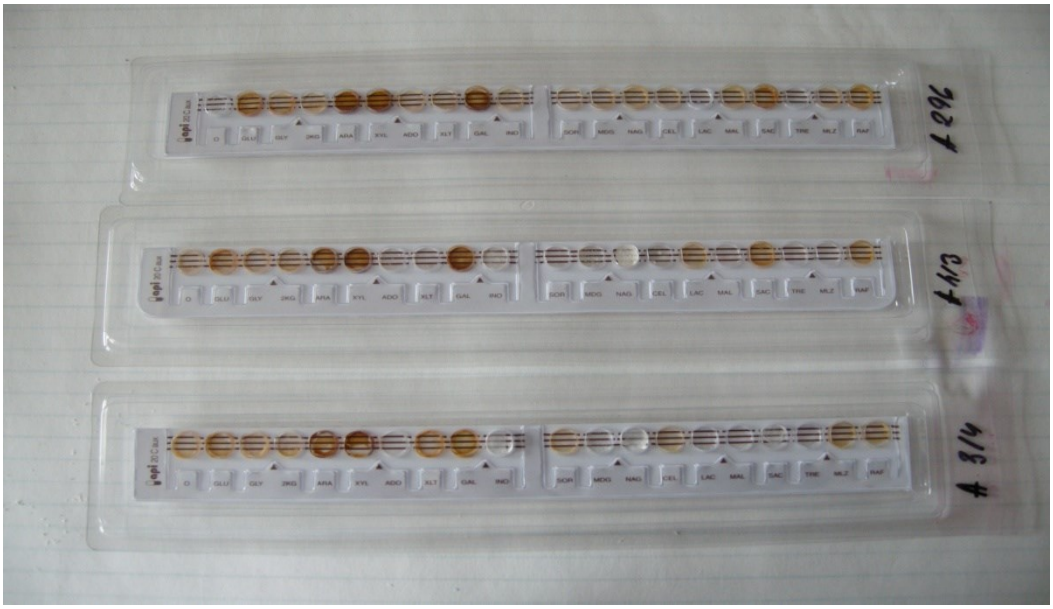

Figure 2. Identifying yeast strains under the acronym A: 314, 413, 296 belonging to the *Saccharomyces* genus, yeasts isolated from must made of grapes from Apold

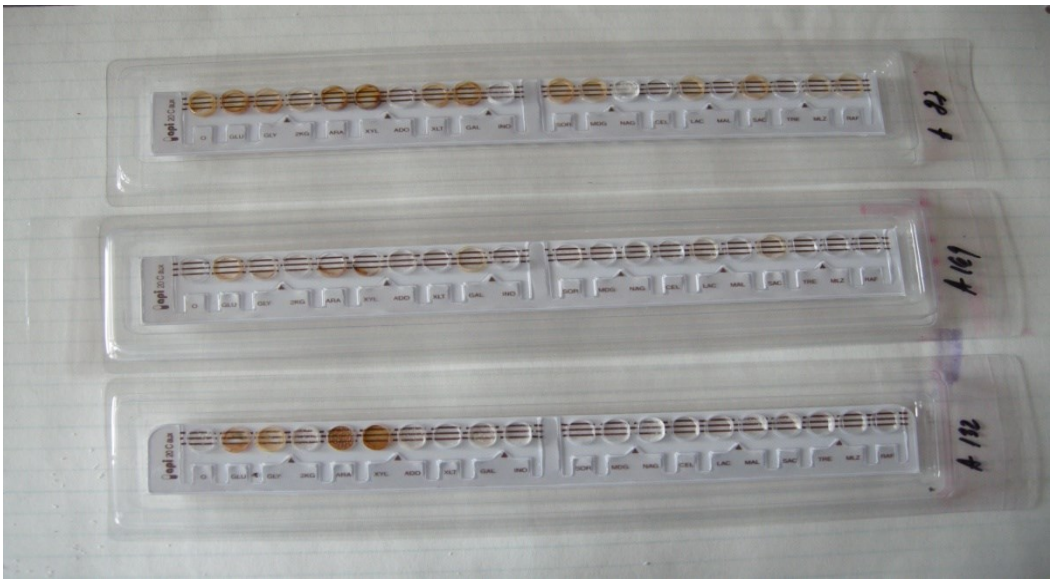

Figure 3. Identifying yeast strains under the acronym A: 132, 169, 87 belonging to the *Saccharomyces* genus, yeasts isolated from must made of grapes from Apold

Fot the purpose of genetic validation, we selected 6 strains, pre-identified through API 20 C AUX tests, under the acronym A: 87, 132, 169, 296, 314, 413.

Table 1. Values of restriction fragments resulting following enzymatic digestion

strains	PCR product(bp)	Restriction fragments		
		*Cfo*I	*Hae*III	*Hinf*I
A 87	880	385/365	320/230/180/150	365/155
A 132	880	385/365	500/220/145	365/155
A 169	880	385/365	320/230/180/150	365/155
A 296	878	385/365	320/230/180/150	365/155
A 314	880	385/365	320/230/180/150	365/155
A 413	878	385/365	500/220/145	365/155

Comparing the resulted fragments with the data in scientific literature (Esteve-Zarzoso et al. 1999), it is ascertained that this method led to the genetic identification of 4 *Saccharomyces cerevisiae* strains, A87, A169, A296, A314 and 2 *Saccharomyces bayanus* strains, A132 and A413. The amplified PCR products presented values of 878 bp -880 bp, which are specific to *Saccharomyces bayanus* and *Saccharomyces cerevisiae strains.*

4. CONCLUSIONS

The identification of wine yeasts specific to wine regions through macro- and microscopic examination must be completed with genetic analysis, thus getting a correct overview of the genera and species under discussion. To a great extent, physiological and morphological traits depend on the microorganisms growth factors; as a consequence, errors may slip into the taxonomic assesment of strains. Using ITS1 și ITS4 primers to amplify restriction sequences and *Cfo*I, *Hae*III și *Hinf*I digestion enzymes, we identified four strains of the *Saccharomyces* genus, *cerevisiae* species and two strains of the *Saccharomyces* genus, *bayanus* species. Viticulturists in the area find the identification of these yeast strains extremely important, as they maintain specific traits, resulting in typical, authentic wines.

API tests are methods which lead to the fast identification of microorganisms, but their results are limited. Using them, we were able to identify 458 *Saccharomyces cerevisiae* yeast strains; nevertheless, genetic tests proved that some of them belong to the species of *bayanus.* In order to avoid errors, we recommend using this method, along with molecular determinations.

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