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Identification of yeasts in early stages of spontaneous fermentation of a red grape must

OANA IFTIME[1], RALUCA GHINDEA, TATIANA VASSU, ELENA SĂSĂRMAN, SIMONA SOARE, ROBERTINA IONESCU
University of Bucharest, MICROGEN Research Center, Aleea Portocalelor 1-3, sector 5, Bucureşti, Romania
[1] corresponding author

Abstract

The main scope of our work was to establish the affiliation of some members of microbial communities involved in traditional wine-making. There were performed a series of tests required for identifying the yeasts participating to early stages of spontaneous fermentation of a red grape must from the Bacau region. The approach was a modern polyphasic one that made use of classical means of identification such as the study of the morphology and physiology of the strains as well as of molecular tests. All data obtained were analysed employing a professional soft especially designed to perform statistic analysis intended to lead to an objective outcome with respect to the established affiliations. Following our study, the four yeast species isolated from grape and must were all identified, as Hanseniaspora occidentalis, Metschnikowia pulcherrima and Saccharomyces cerevisiae (two different strains).

Keywords: wine yeasts, spontaneous fermentation, polyphasic taxonomy, numerical taxonomy, *Hanseniaspora occidentalis, Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*.

Introduction

In the last few years the interest towards microbial communities involved in the obtainment of traditional alimentary products arose significantly [2; 3; 7; 14]. This is due both to the fact that producers try to offer novel 'temptations' to the public in order to gain some advantage on the market and to the fact that consumers are more and more claiming for ecological feed and beverages with no chemicals added.

Wine industry represents a quite conservative branch of agriculture. Refined blends are produced up to now by traditional methods that make well kept secrets in every famous winery and identification of the participating microbial strains is avoided on purpose. On the other hand, large scale production of ordinary wines is continuously developing and research in the field of grapes, must and wine ecology as well [9; 15; 18; 19; 24]. Identification of valuable wild strains with superior qualities is the main expected outcome of such research [11; 13].

Various molecular methods can be employed in order to establish affiliation of yeasts known to be present in media were alcoholic fermentation takes or has taken place [5; 8; 12; 16].

Still, when identification of newly isolated microorganisms is concerned, classical morpho-physiological tests are often combined with molecular techniques, in a polyphasic

perspective that has the advantage of enhanced accuracy even though it is more laborious [4; 6; 10; 20].

We also used a polyphasic approach in conjunction with a numerical taxonomy method in order to establish affiliation of four yeast strains isolated from red grapes and must.

Materials and methods

Microbial strains and cultivation

The following microbial strains were used: four wild strains isolated from grapes and must from the Bacau region, Romania, namely ODA, yeast strain isolated from grapes, not found in fermented must, ODM, yeast strain isolated from fermented must, ODR, yeast strain isolated from grapes, also present in must at the very beginning of fermentation, ODS, yeast strain isolated from fermented must and associated with bacteria (lactic bacteria?).

All strains were maintained on agar YPG (Yeast- Peptone-Glucose, glucose:20 gL^{-1}, peptone 10 gL^{-1}, yeast extract 5 gL^{-1}, agar 20 gL^{-1}). According to experimental conditions yeasts were also grown on other media, as needed: on agar YNB (DIFCO) while assessing the ability to use organic compounds as sole source of carbon for aerobic growth, on YE (yeast extract 5 gL^{-1}) during the assessment of the ability to use certain sugars anaerobically, on urea broth (peptone $1gL^{-1}$, glucose $1gL^{-1}$, NaCl 5 gL^{-1}, KH_2PO_4 $2gL^{-1}$, phenol red 0.012 gL^{-1}, urea 40 gL^{-1}) while performing the urease test, on YPG for DNA isolation.

Microscopical examination and physiological tests

Non- filamentous vegetative cells were examined under microscope. The four wild strains were tested for filamentous growth by the method described in Barnet et. al [1]. Four physiological tests were performed, all by the methods described in Barnett et. al [1]: production of extracellular starch- like compounds, assessing the ability to use organic compounds as sole source of carbon for aerobic growth, assessing the ability to use certain sugars anaerobically *and the* urease test.

Data analysis

Results of the physiological tests served as variables for statistic analysis. Analysis was performed by the *unweighted pair group average method (UPGA),* frequently employed in numerical taxonomy. UPGA is a cluster analysis method from the 'Joining (tree) clustering' category in which the distance between two clusters is calculated as the average distance between all pairs of objects in the two considered clusters [17; 25; 26].

Because of the high variability showed by *Saccharomyces cerevisiae* in response to physiological tests random combinations of data characterizing hypothetical reference strains were generated in series of ten strains each. Those series were used one by one in order to compare data obtained for strains ODM and ODS. ODM and ODS were simultaneously compared to variants of *Saccharomyces exiguus*, another yeast known to be present in grape must whose morphology is quite similar to that of *Saccharomyces cerevisiae*.

The same procedure was applied in the case of ODA and ODR but to a smaller extent as the number of possible combinations of data typical for the species to which ODA and ODR might have belonged was not that large as in the case of *Saccharomyces cerevisiae*.

Data obtained for ODA were compared to all possible variants valid for *Hanseniaspora guilliermondii, H. occidentalis, H. osmophila, H. uvarum, H. vineae* that can be found with grapes and also for *H. valbyensis*, found in soil. Strains from genus *Hanseniaspora* were elected as references because of the typical lemon shape of ODA's cells.

Data obtained for ODR were compared to all possible variants valid for *Metschnikowia pulcherrima*, found on grapes, as ODR produced a characteristic red pigmentation indicating it might have belonged to that species.

Chromosomal DNA extraction and purification

Chromosomal DNA was extracted from ODA by the method described by Vassu et al. with a series of modifications [22]. A volume of 1.5 mL of a late-log-phase culture was centrifuged at 7,000 rpm for 7 minutes. The pellet was re-suspended in 375 μL TEG (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose; pH 8.0) with 2μl β- mercaptoethanol subsequently added and incubated at 37^{o}C for 30 min. 200 μL of 2 mgmL^{-1} zymoliase was added and the samples were incubated at 37^{o}C for 90 min. Samples were centrifuged at 6,500 rpm for 8 min the cells were resuspended in 300 μL TEG + 33 μL 1% sodium dodecyl sulphate (SDS) + 3μL 20 mgmL^{-1} proteinase K and incubated at 37^{o}C for 30 min. 2μL 2.5 M KCl was added, the samples were incubated at room temperature for 10 min then centrifuged at 12,000 for 12 min. For half of the samples, 3 μL 10 mgmL^{-1} RNase was added to supernatant, the mixture was incubated at room temperature for ten minutes and centrifuged at 12,000 rpm for 12 min. Chromosomal DNA was extracted twice with CIA (chloroform- isoamilic alcohol 24:1, vol/vol), precipitated with isopropanol and incubated for 10 min at room temperature. After a subsequent 15 min centrifugation at 14,000 rpm and a 15 min incubation at room temperature chromosomal DNA was re-suspended in 40 μL TE pH 8,0 (10 mM Tris-HCl, 1mM EDTA). The samples intended to be used for determination of the guanine+ citosine content were re-suspended not in TE but in 20 μL SSC [1X] (the SSC [10X] solution contains 0.15 M NaCl, 0.015 M trisodium citrate). Concentration and purity of the extracts was estimated spectrophotometrically and also by agarose gel electrophoresis.

Determination of guanine-plus-citosine content

The guanine- plus- citosine content that was required in order to clarify the ODA strain's affiliation was determined by thermal denaturation. Genomic DNA was re-suspended in SSC (0.15 M NaCl, 0.015 M sodium citrate) buffer. The temperature of the mixture was increased slowly (1^{o}Cmin^{-1}) from 20^{o}C up to 100^{o}C and the absorbance of the solution at 260 nm was continously monitored spectrophotometrically. The melting temperature (Tm) of DNA was defined as the temperature at 50% hyperchromicity. Tm was needed in order to calculate the G+C content with Owen's formula: % G+C = 2.08○ Tm – 106.4 [21].

Results

Data analysis

Results of physiological tests were designated as variables for statistic analysis. A numerical value (*e.g.* 1 for positive, 2 for negative) was assigned to each possible variant of a considered variable. A few examples were given in **Table 1** and **Table 2**.

Clustering by UPGA revealed affiliation of the ODR strain to *Metschnikowia pulcherrima* (**Fig. 1**).

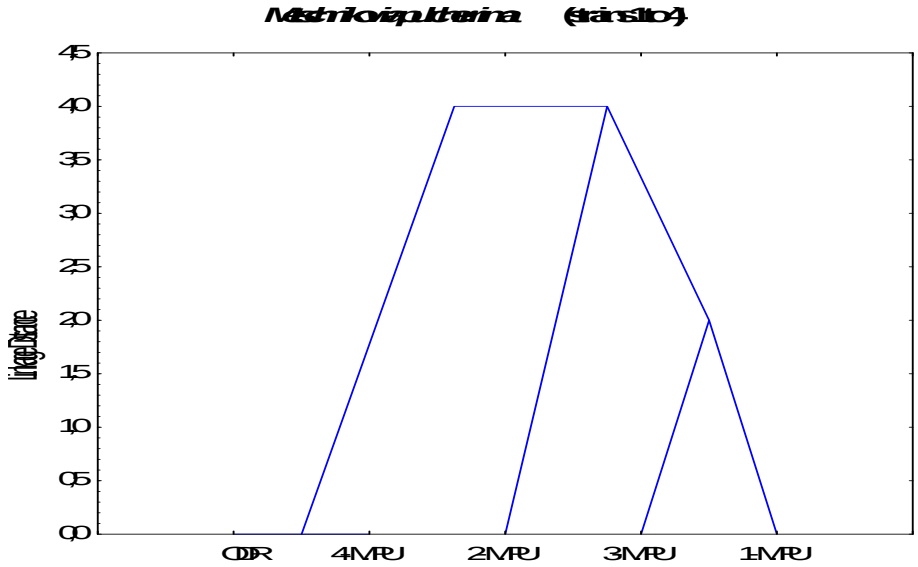

Fig. 1. Identification of the ODR strain as a member of the *Metschnikowia pulcherrima* species by the UPGA method

According to the UPGA analysis ODM and ODS were finally found to belong to the *Saccharomyces cerevisiae* group of strains as can be seen in **Fig. 2** and **Fig. 3**, respectively. ODM data matched those typical for reference strain *Saccharomyces cerevisiae- 37* from series no. 4, while ODS' data matched characteristics of *Saccharomyces cerevisiae- 102* numbered among strains in series 11.

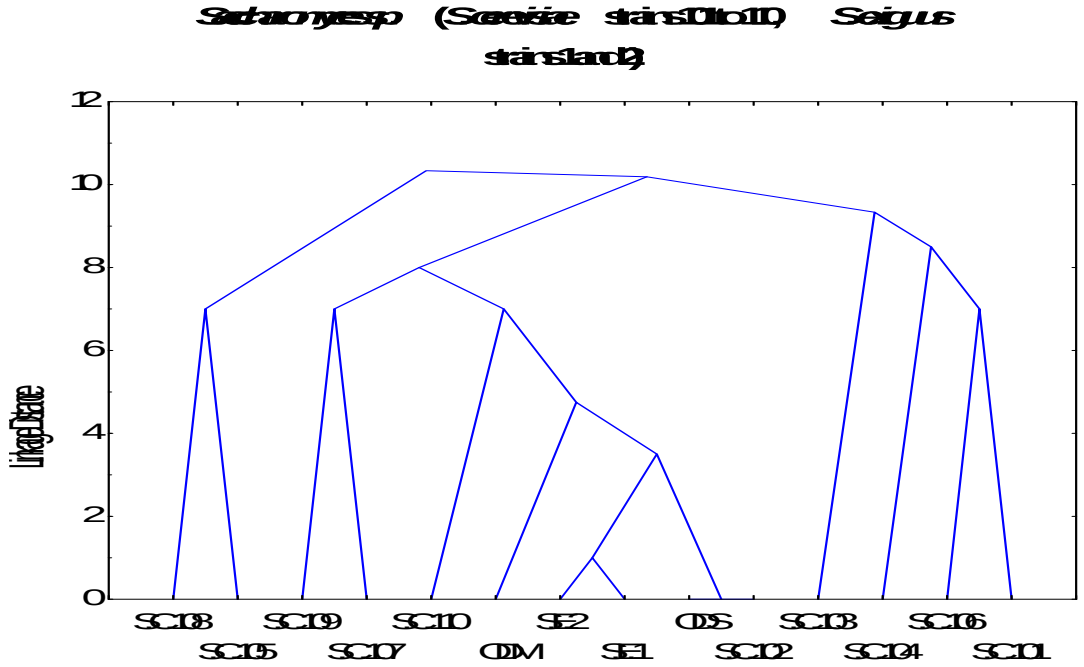

Fig. 2. Identification of the ODS strain as a member of the *Saccharomyces cerevisiae* species by the UPGA method (correspondence with the *S. cerevisiae- 102* randomly generated pattern)

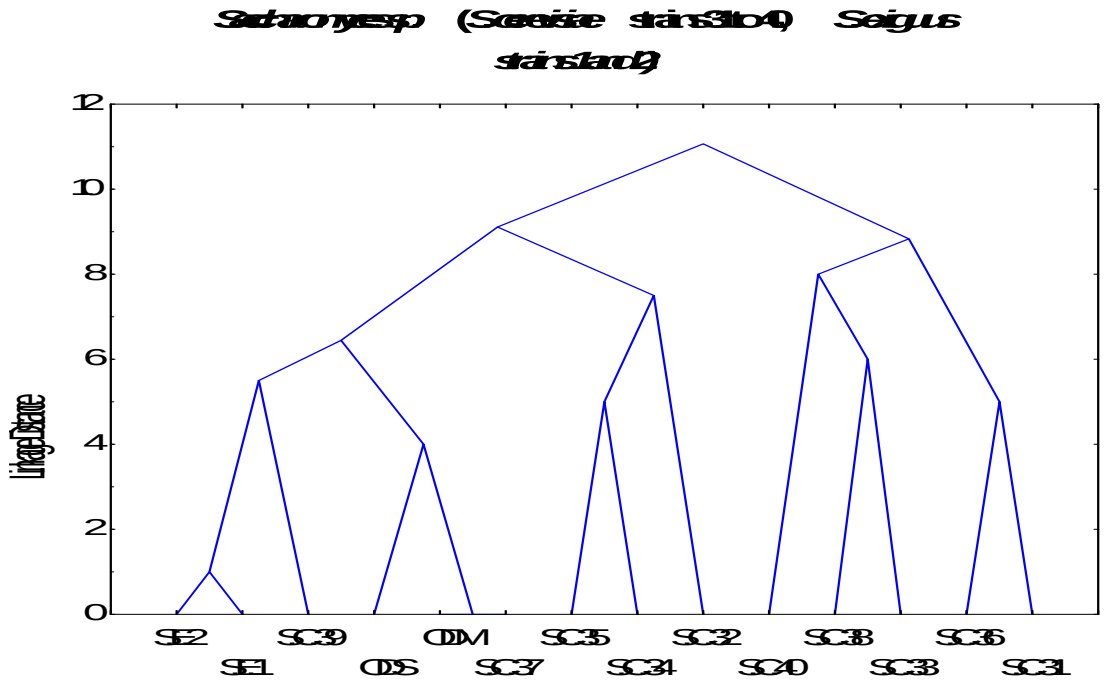

Fig. 3. Identification of the ODM strain as a member of the *Saccharomyces cerevisiae* species by the UPGA method (correspondence with the *S. cerevisiae- 37* randomly generated pattern)

Affiliation of the ODA strain remained unclear till determination of G+C content. It might as well have belonged to any of the species in genus *Hanseniaspora* that it was compared with (**Fig. 4**).

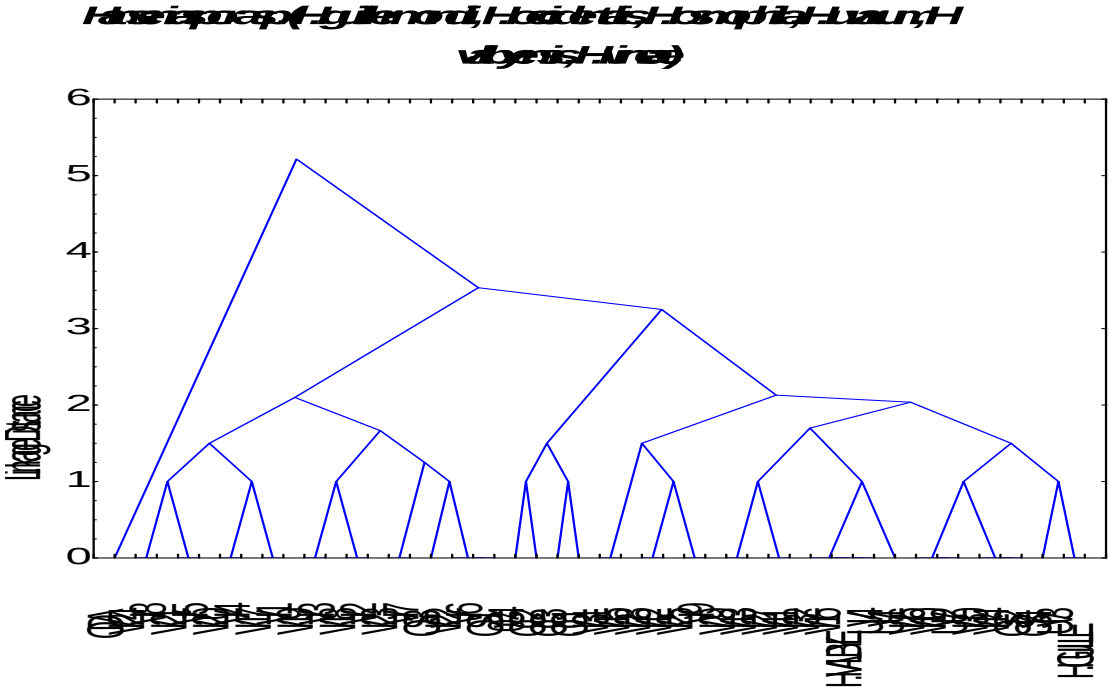

Fig. 4. Identification of the ODA strain as a member of the *Hanseniaspora* genus by the UPGA method

Chromosomal DNA extraction and purification

DNA extracts of very good quality were obtained thus allowing guanine-plus-citosine content determination.

Determination of guanine-plus-citosine content

This type of analysis was of particular use in the case of the ODA strain that exhibited a 35,8 G+C content which allowed us to conclude that it belongs to the *Hanseniaspora occidentalis* group of strains involved in spontaneous alcoholic fermentation of must.

Discussion

Using a minimum of tests, ODA was found to be part of the *Hanseniaspora occidentalis* species, ODR was found to belong to *Metschnikowia pulcherrima*, ODM and ODS were both revealed as members of the *Saccharomyces cerevisiae* large group of strains involved in spontaneous alcoholic fermentation of must.

In the case of the ODA strain neither morphological and physiological tests (classical tests) could not be solely employed in order to establish beyond doubt the affiliation of the newly isolated microbial strain.

Establishing the affiliation of the wild strain by a polyphasic approach in conjunction with numerical taxonomy led to very reliable results.

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