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

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Abstract

Little is known about the yeast strains involved in spontaneous fermentation of the musts used in Romanian country-side households for obtaining wine. The present work aimed to lead to the identification of such strains isolated from a fermented red grape must from Bacău region. In order to establish the affiliation of three strains that were found to be typical for late stages of fermentation there were performed a series of morphological, physiological and also molecular tests. Following analysis of the obtained data by objective, statistical means two of the strains were positively identified as Saccharomyces cerevisiae while the third strain was found to belong to the genus Candida, species Candida vini, a spoilage yeast.

Keywords: wine yeasts, spontaneous fermentation, polyphasic taxonomy, numerical taxonomy, *Candida vini, Saccharomyces cerevisiae*

Introduction

Yeasts that can be present in late stages of spontaneous fermentation of grape musts may belong to a few different genera and species- *Sacharomyces sp.*, and other genera and species with representatives acting as spoilage yeasts. Among the latter it should be mentioned *Candida sp (C. rugosa*, *C. vini*, *C. zeylanoides., Dekkera (anamorph Brettanomyces) bruxellensis., Issatchenkia orientalis (syn. Candida krusei*), *Pichia membranefaciens*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, and *Zygosacharomyces bailii* [7]. Also, *Saccharomyces* wine strains exhibit a huge diversity. The same ecological niche, namely the fermented must may host a few different strains, according to the stage of fermentation (early fermentation *vs.* late fermentation strains) being also possible that more than one strain be present in the must at the same moment [1].

Microbial communities involved in the obtainment of traditional alimentary products elicited great interest in the last decade [3; 8].

Methods of identification of such isolates down to the strain level are still deficitary with respect to their accuracy. A satisfactory identification of the yeasts down to the species level may however be obtained using either “classical” (morphophysiological), molecular (DNA analysis) or a combination of both types of methods also known as the “polyphasic” approach [4; 5; 6; 7]. Numerical taxonomy is very useful in establishing the affiliation of newly isolated wild yeast strains mainly because it introduces a certain degree of objectivity.

In the attempt to identify the three yeast strains isolated from a late stage of spontaneous fermentation of a red grape must we employed a combination of methods, in a polyphasic perspective and also a a numerical taxonomy tool that integrated obtained data into a coherent final diagnosis of the studied strains.

Materials and Methods

Microbial strains and cultivation

The following microbial strains were used: three wild strains isolated from fermented must from Bacau region, Romania, namely GMD-C, GMD-N, GMD-T all isolated from a late stage of spontaneous fermentation of the product.

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 [2]. Four physiological tests were performed, all by the methods described in Barnett et. al [2]: *production of extracellular starch- like compounds, phylamentous vs. non- phylamentous growth, assessing the ability to use organic compounds as sole source of carbon for aerobic growth* (15 carbon sources)*, assessing the ability to use certain sugars anaerobically* (11 sugars) and the *urease test*.

Data analysis

Analysis of the results of the morpho-physiological tests was conducted *via* the *unweighted pair group average method (UPGA)*, which is a numerical taxonomy tool. 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 [9; 13; 14]. Null linkage distance (no connecting branch) between two strains indicates a 100% similarity - the identical strains will appear as all placed on the OX axis. Strains that differ from each other will be grouped via branches expressing the corresponding degree of similarity, lower that 100% in such cases.

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 GMD-N and GMD-T. GMD-N and GMD-T were simultaneously compared to variants of *Saccharomyces exiguus*, another yeast known to be present in grape must and morphologically similar to *Saccharomyces cerevisiae*.

Data obtained for GMD-C were compared to all possible variants valid for *Candida boidinii* and *Candida vini*, the two possible affiliations of GMD-C as indicated by the first observations' results (the abiltiy to develop false hyphae both under aerobic and anaerobic conditions) and data from literature on the *Candida* species to be found in fermented grape musts.

Genomic DNA extraction and purification

Genomic DNA was extracted from GMD-C by the method described by Vassu et al. with a series of modifications [11]. A volume of 1.5 mL of a late-log-phase culture was centrifuged at 7,000 rpm for 7 minutes. The pellet was resuspended 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. 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 resuspended 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 resuspended 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. In order to investigate the presence of RNA (killer) plasmids the samples were not treated with RNase and the agarose gel was loaded with DNA + BFE (bromphenol blue 0.4%, sucrose 55%; i.e. loading solution without RNase).

2µm plasmid isolation and purification

The 2 μm plasmid was isolated according to the method described by Vassu et al. (2000) [12]. Biomass obtained by cultivation on YPG was collected by centrifugation and treated with 1 mL TE-1 (Tris 0.1 M, EDTA 0.005 M, pH 9.3), 20 μL β-mercaptoethanol, 1mL CFEM (citric acid 0.021%, Na_2HPO_4 0.141%, EDTA 3.72%, mannitol 18.21%, pH 5.8) for 25- 30 μL biomass and a cell wall breaking enzyme in order to obtain protoplasts. Protoplasts were destroyed and DNA denaturated with a solution of SDS 1% plus NaOH 0.2 N pH 12.5 (400 μL per sample). Plasmidial DNA was selectively renaturated by adding 300 μL potassium acetate tampon solution (pH 4.8). Plasmidial DNA was purified with a mixture of solvents (chloroform: isoamylic alcohol 24:1) and precipitated with isopropanol. The DNA sediment was dissolved in TE (Tris 10 mM, EDTA 1mM, pH 8.0).

Results

Data analysis

In order to perform the UPGA test, data gathered from physiological tests were designated as variables (the traits, e.g. ability to use a certain sugar) and variants or states (e.g. positive- to which the numerical value 1 was attached or negative- to which it was attached the numerical value 2), respectively. For example, the ability of strains to grow on D- glucose was coded as variable no. 1 with two possible variants: able to grow on D-Glucose- positive- 1 and unable to grow on D- glucose- negative-2. Some more examples are presented in **Table 1** and **2**.

Table 1. Exemplification of the assignment of numerical values to some variables employed in the UPGA method applied to wild yeast strains isolated from grape and must. Physiological traits- ability to use certain sugars anaerobically

	PHYSIOLOGICAL TRAITS **Ability to use certain sugars anaerobically**					
	Glucose	Galactose	Maltose	Me-α-D-glucoside	Sucrose	Trehalose
Variable no.	1	2	3	4	5	6
Variants	1- + 2- -	1- + 2- -	1- + 2- -	1- + 2- -	1- + 2- -	1- + 2- -
GMD-C	1	2	2	2	2	2
GMD-N	1	1	1	2	1	2
GMD-T	1	1	1	2	1	2

Table 2. Exemplification of the assignment of numerical values to some variables employed in the UPGA method applied to wild yeast strains isolated from grape and must. Physiological traits- ability to use certain sugars anaerobically (continued)

	PHYSIOLOGICAL TRAITS **Ability to use certain sugars anaerobically**				
	Melibiose	Lactose	Celobiose	Melezitose	Raffinose
Variable no.	7	8	9	10	11

Variants	1- + 2- -	1- + 2- -	1- + 2- -	1- + 2- -	1- + 2- -
GMD-C	2	2	2	2	2
GMD-N	2	2	2	1	1
GMD-T	2	2	2	1	1

Strains GMD-N and GMD-T were both identified as *Saccharomyces cerevisae*, exhibiting zero linkage distance from the same reference strain *Saccharomyces cerevisiae-127* (**Figure 1**). This indicates they are identical to that precise strain and also to each other.

Strain GMD-C was found to belong to the genus *Candida*, clustering to the species *Candida vini* (**Figure 2**).

Discussion

Using only few tests, the GMD-N and GMD-T strains were identified as *Saccharomyces cerevisiae*, a species with many different members involved in all stages of spontaneous fermentation of grape musts. A question remained to be answered, namely if it was the same strain, isolated twice or not. Some molecular tests were performed, with a multiple aim- to investigate further the identity between the strains and other traits typical to wild wine yeasts in general and to *Saccharomyces cerevisiae*, respectively. No differences between the two strains could be noticed as resulting from those supplemental tests. Both strains lack killer plasmids (**Figure 3**) and both have the 2 μm plasmid (**Figure 4**).

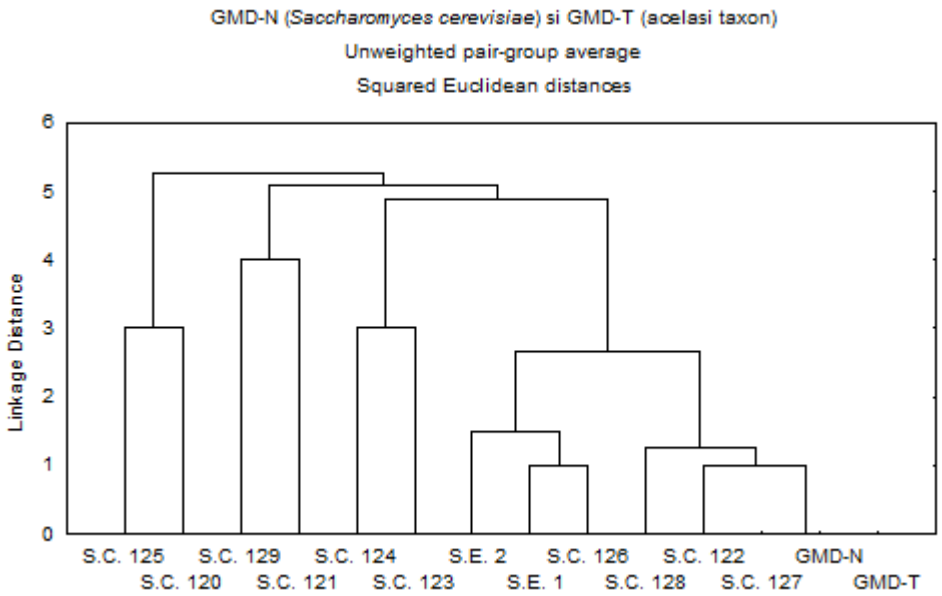

Figure 1. Identification of the GMD-N and GMD-T strains as members of the *Saccharomyces cerevisiae* species by the UPGA method. Both strains exhibit zero linkage distance to the same reference.

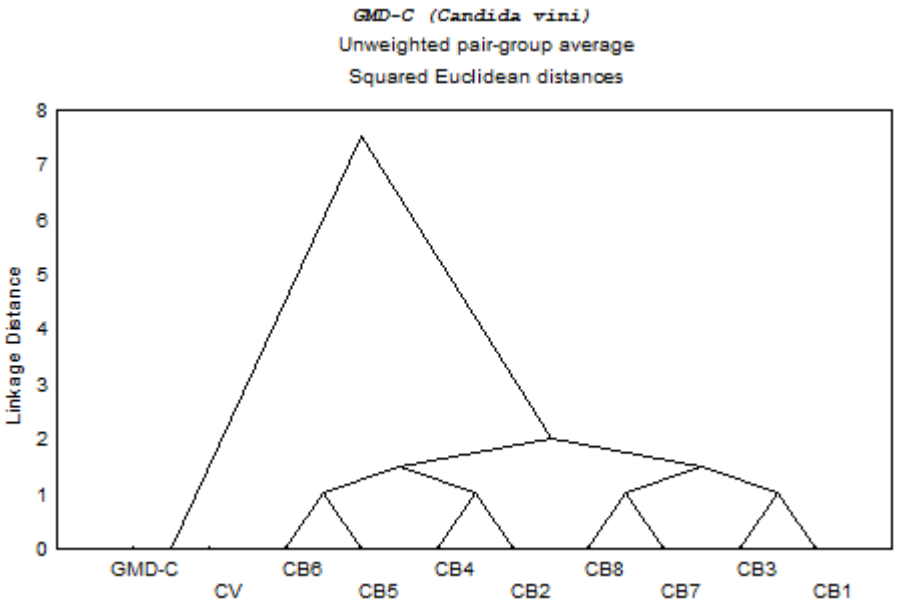

Figure 2. Identification of the GMD-C strain as a member of the *Candida vini* species by the UPGA method

Figure 3. Electrophoresis of genomic DNA extracts from GMD-N and GMD-T (genomic DNA extraction method, samples not treated with RNase). Line 1- GMD-N, line 2- GMD-T

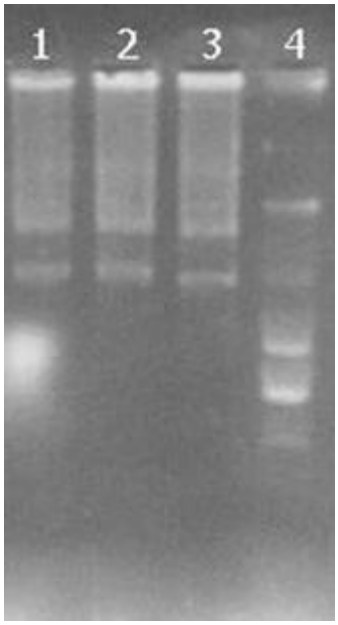

Figure 4. Electrophoresis of DNA extracts from GMD-N and GMD-T (the 2µm extraction method) Line 1- *S. cerevisiae* D649 (reference strain), traces of chromosomal DNA and the 2 µm plasmid; line 2- GMD-N, traces of chromosomal DNA and the 2µm plasmid; line 3- GMD-T, traces of chromosomal DNA and the 2µm plasmid; line 4- λ/BstII, molecular weight marker

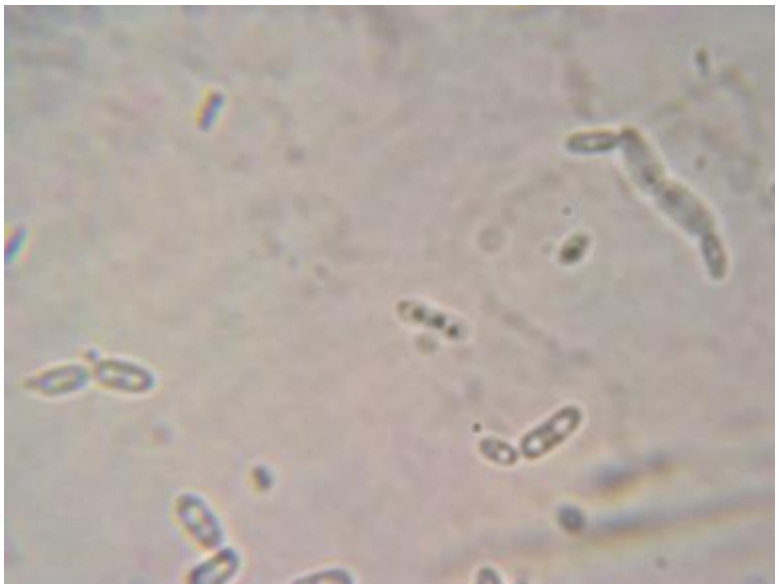

Figure 5. The elongated cells and phylamentous growth of GMD-C (*Candida vini*)

The third isolate, GMD-C was identified *via* the combined numerical taxonomy- morpho-physiological testing approach as belonging to the species *Candida vini*. Still, the strain shows a particular morphology. *Candida vini* is supposed to have its cell morphology similar to that of *Saccharomyces cerevisiae*, with spherical or ovoidal cells [2]. Our strain had elongated cells (**Figure 5**).

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