Indian Journal of Experimental Biology
Vol. 58, July 2020, pp. 461-473

Isolation and characterization of yeast strains from Badacsony, Hungary

Annamária Gerőcs[1], Katalin Nemes-Barnás[1], Sára Pál[1], Barna Szőke[2], János Májer[2], Tibor Farkas[1]*# & Ferenc Olasz[1]*

[1]Agricultural Biotechnology Institute, National Agricultural Research and Innovation Centre (NARIC ABC), Szent-Györgyi Albert Street 4. H-2100 Gödöllő, Hungary

[2]Research Institute for Viticulture and Oenology, National Agricultural Research and Innovation Centre - (NARIC RIVE) Római Street 181. H-8261 Badacsonytomaj, Hungary

Received 14 June 2019; *Revised 04 May 2020*

In modern winery, starter strains are used for wine making to avoid the risk of slow or incomplete fermentation. However, application of commercial starter yeasts sometimes leads to a uniform character of the wines. On the other hand, indigenous ("terroir") strains are adapted better to local conditions highlighting the specific taste of wine. In this study, we isolated local yeast strains from Badacsony wine region of Hungary and investigated with microbiological and molecular biological tests in order to develop indigenous starter selection method. As many as 480 yeast strains were isolated and grouped using carbohydrate and nitrogen sources. Finally, 80 selected isolates were characterized for important oenological features, including tolerance of glucose, ethanol and acetic acid. Fermentation ability, killer toxin, hydrogen sulfide and acid production of 80 selected isolates were also tested. Isolates were studied by applying two molecular methods based on rRNA gene sequencing and analysis of Ty retrotransposon's delta elements in case of *Saccharomyces* strains. Our results have shown that the isolated strains belong to 15 yeast species of 8 genera, and the diversity of yeast population was significantly high in the investigated vineyard. We have found that selection for technological properties was a potential way to find suitable strains from the local microbiome, because a high proportion of isolated wild yeast strains show beneficial oenological properties for wine making. Further, we studied 35 available starter yeasts to avoid re-isolation and we identified only 3 starter yeasts from grape and must samples, which can be considered as very low incidence.

Keywords: Badacsony wine region, Grapes, *Kéknyelű* grape variety, Must microflora, *Saccharomyces*, Starter yeast selection, Terroir yeasts

Badacsony wine region is located on the northern shore of Lake Balaton and it is one of the most famous wine making areas of Hungary. The sub-Mediterranean climate of this region and the soil, which is a mixture of clay, loess, and sand on top of volcanic basalt, is ideal for grape cultivation. Several grape varieties are cultivated in Badacsony, for example "Olaszrizling", "Szürkebarát", "Tramini", "Kéknyelű". The relatively rare white variety "Kéknyelű" is cultivated only in Hungary, and it has emerged as the autochthonous grape and wine of the region.

Different wine regions are also characterized by their specific microbial communities, which are adapted to the local environmental conditions. These species play a decisive role in the local spontaneous wine fermentation. Many genera and species of yeasts (*Saccharomyces* and non-*Saccharomyces*) are present in the grape-juice and the composition of the yeast population changes during fermentation due to increasing ethanol level[1]. Non-*Saccharomyces* yeasts are generally dominant in the early and middle stages of fermentation, but in the final stage of the fermentation process *Saccharomyces* strains emerge[2]. The indigenous "terroir" yeasts can produce several compounds of wine that adds to the wine's specific character[3,4], for eg. alcohols, organic acids, aroma compounds, etc[5]. It is known that the important feature of wine, the aromatic compounds, are configured by yeasts and bacteria during fermentation and these compounds are synthesized mostly in post-fermentative stages. Different yeast species can produce pectinolyzic enzymes, which degrade grape skins and help to extract the colour and flavour components. According to Arora & More[6], commercial pectolytic enzymes decrease fermentation time but as an alternative approach, there are also some pectinolytic wine yeasts which can be applied instead of purified enzymes[7,8].

The organoleptic parameters of wine are dependent on the inoculated starter yeast[9], which in general, is

*Correspondence:
Phone: +36 28526240; Fax: +36 28526198
E-mail: olasz.ferenc@abc.naik.hu (FO), tibor.farkas@ceva.com (TF), gerocs.annamaria@abc.naik.hu (AG)

Saccharomyces cerevisiae strain. Selection of commercial strains is based on their beneficial properties: rapid fermentation, high alcohol tolerance, low synthesis of hydrogen sulfide, tolerance of sulfuric acid, production of glycerol and low production of foam[10]. Use of commercial starter strains can reduce wine complexity due to decreasing biodiversity in the musts and hide the character of local wines[11]. This justify the ample literature available on isolation and characterization of indigenous yeast strains and selected for wine fermentation. Guimarães *et al*.[12] isolated *S. cerevisiae* strains from Colombo in Brazil. *S. cerevisiae* species were identified by the carbon and nitrogen assimilation tests, fermentative capacity test and PCR assay. Similarly, Regodón and colleagues[13] collected fermenting musts and wines from wineries located Extremadura (Spain) and isolated 86 local *S. cerevisiae* strains selected for resistance to sulfur dioxide, afterwards they tested for killer toxin and foam production, tolerance for high temperatures and the selected strains were used in vinification. Csoma *et al*.[14] isolated *Saccharomyces* yeasts from four wine regions in Hungary and analyzed those using genotypic and phenotypic methods.

Combination of *S. cerevisiae* with different non-*Saccharomyces* strains (*Kluyveromyces, Candida*) is also know to enrich aroma complexity of the final product[15-17]. Different inoculation methods have also been tested such as mixed preparation, which means non-*Saccharomyces* and *Saccharomyces* strains added at the same time. In other cases, non-*Saccharomyces* strains is used as single cultures to start fermentation and after few days must samples are inoculated with a *Saccharomyces* strain[18-20].

In this study, we focused on collecting data about the diversity of natural yeast community in a selected vineyard located in Badacsony wine region and also selection of beneficial strains for wine making. Very little information is available about the yeast community of the Badacsony[14] and that of the most representative grape variety "Kéknyelű". Here, we attempted to isolate and identify "terroir" yeast strains from different sources (from grape berries, grape juice and from different phases of must fermentation) in order to describe the community of culturable yeasts. We intend to select and characterize some strains using different physiological tests, which allow sorting out potentially starter strains for wine fermentation.

Materials and Methods

Sample collection and isolation of yeast strains

Yeast strains were isolated from the NARIC RIVE vineyard in the Badacsony wine region (Badacsonytomaj) in September and October 2014. The following sample types were collected: grape berries of the Hungarian variety "Kéknyelű", grape juice and must from early, middle and final phases of spontaneous wine fermentation. Must extraction and wine fermentation were performed under laboratory conditions. The surface of the grape berries was washed with 0.8% NaCl solution. All kind of samples were plated onto YPD solid media (1% yeast extract, 2% peptone, 2% dextrose and 1.5% agar) and incubated at 30°C for 48 h under aerobic conditions. Media were supplemented with antibiotics and antifungal agent to inhibit the growth of moulds and bacteria[21,22] in the following concentrations: ampicillin (Duchefa, Netherlands) 150 μg/mL, chloramphenicol (Duchefa, Netherlands) 20 μg/mL and biphenyl (Sigma-Aldrich, USA) 150 μg/mL. The isolated strains were grown in YPD liquid medium and after growth, glycerol was added at the final concentration of 37% to every sample as cryo-protectant agent and samples were stored at −70°C.

In this study, 35 different commercially available dry yeast starter cultures were used as references. Starter yeasts were denominated as: YS28, YS33, YS34, YS36, YS37, YS38, YS42, YS45, YS46, YS47, YS48, YS49, YS50, YS51, YS52, YS53, YS54, YS55, YS56, YS57, YS58, YS59, YS60, YS61, YS62, YS63, YS64, YS65, YS66, YS67, YS68, YS69, YS70, YS71 and YS72. Dry yeasts were grown in YM (6 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract, 10 g/L glucose) liquid medium at 30°C overnight before use.

Selection of isolates according to utilization of carbohydrate (CA) and nitrogen sources (NA) for investigation

Inoculum preparation

Yeast isolates were grown overnight in YM medium at 30°C. Two mL aliquots of each sample were harvested by centrifugation at 5000 rpm for 5 min and the supernatant was removed. The pellet was washed with sterile 0.8% NaCl solution for three times to remove components of the medium. Pellets were dissolved in 0.8% NaCl solution. The optical density (OD) of yeast cells suspension was adjusted between 0.1-0.2 at 600 nm before microbiological tests.

Carbohydrate assimilation test

About 480 isolates were tested on nitrogen base minimal agar (HiMedia, India) containing different

carbohydrates (2%): glucose, sucrose, galactose, mannose, xylose, arabinose, maltose, glycerol, rhamnose, cellobiose, melibiose. Plates were incubated at 30°C for 48 h[23]. Carbohydrate free agar was used as negative control. Presence of colonies on the plates after 2 days indicated that the corresponding yeast was able to assimilate the respective carbohydrate. Yeast isolates were grouped based on their carbohydrate assimilation pattern.

Nitrogen assimilation test

The 480 isolates were plated onto carbon base minimal agar (HiMedia, India) supplemented with different nitrogen sources (1%): ammonium sulfate, lysine, tryptophan, sodium nitrite, potassium nitrate and incubated at 30ºC for 48 h. Nitrogen free agar was used as negative control. Growth of colonies on the plates indicated the ability of the yeast strains to utilize the corresponding nitrogen source[23]. Based on the results, groups were configured.

Phylogenetic identification of yeast strains

The selected 480 strains were plated onto YM medium and incubated at 30°C to obtain single colonies. One colony of each strain was used as a template in PCR reaction. The ITS region was amplified with primers ITS5 (GGAAGTAAAAGTCG TAACAAGG) (forward) and ITS4 (TCCTCCGCTTA TTGATATGC) (reverse)[24]. The amplification reaction was performed in a total volume of 20 µL consisting of 10 µL DreamTaq PCR mix (catalog number K1071, ThermoFisher Scientific, USA), 0.5 µL (10 µM) of each primer and 9 µL nuclease-free water. The mixture was subjected to an initial denaturation step of 10 min at 94°C, followed by 30 cycles consisting of 30 s at 94ºC, 30 s at 52ºC and 1 min at 72ºC, and a final extension step of 10 min at 72ºC. The products of the PCR were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide, visualized by UV transillumination and documented by Gel Doc XR+ (Bio-Rad, USA). The samples were grouped according to the length of PCR fragment.

Species identification was confirmed by determining and analyzing the nucleotide sequence of the ITS fragment (Biomi Kft., Hungary). At least two sequences representing each group were analyzed by the BLAST program[25]. Phylogenetic tree was constructed using the program Mega7 based on UPGMA cluster analysis[26].

Characterization of selected yeast strains

Inoculum preparation was described previously in Materials and Methods. Studied isolates were selected from groups of carbohydrate assimilation (CA) and nitrogen utilization (NA) randomly.

Ethanol tolerance test

About 80 isolates (40 CA and 40 NA) were plated onto YPD agar supplemented with 5, 10 and 15% v/v ethanol[27] and incubated at 30ºC for 48 h. After 2 days incubation, plates were checked for the presence of yeast colonies.

Glucose tolerance

The selected 80 isolates (40 CA and 40 NA) were studied on YPD agar (without dextrose) in the presence of 100, 200, 300 g/L (10, 20, 30%) glucose. Ekunsanmi & Odunfa's protocol[28] was modified with the following way: we have used solid medium instead of liquid medium, different concentrations and different detection methods. The samples were incubated at 30ºC for 48 h. Tolerant strains were capable of growing after the incubation.

Acid production and acetic acid tolerance test

Overnight cultures of 80 yeasts isolates (40 CA and 40 NA) were plated onto YPD agar supplemented with 5% glucose and 0.5% $CaCO_3$ (acid production test) or 10% glucose and 1% acetic acid (acid tolerance test) and incubated at 30ºC for 48 h[23]. Growth of colonies indicated acetic acid tolerance in the acid production test, while the presence of clear zone around the investigated isolates indicated acid production of yeasts.

Fermentation test

The 80 selected yeast isolates (40 CA and 40 NA) were grown overnight in YM medium at 30ºC. The isolates were inoculated into Durham tubes containing phenol red broth media (1% glucose, 2% yeast extract, 0.0016% bromophenol purple indicator) and incubated at three different temperatures (26, 21 and 16ºC) for 7 days. Glucose supplemented and glucose-free medium inoculated with a commercially available starter culture was used as positive and negative control, respectively. The change of colour and gas (CO_2) production in the Durham tubes indicated fermentation ability of isolates[29].

Killer toxin production

Killer toxin production was determined using a methylene blue containing YEPD medium as described by Izgu and colleagues[30]. Killer sensitive *S. cerevisiae* strain was inoculated on the surface of agar plates and afterwards the studied 80 yeast strains were plated onto the agar medium. Plates were incubated at 24ºC for two days. Killer activity was indicated by clear zones around the studied strain and the presence of blue staining indicated death cells.

Hydrogen sulfide (H_2S) production

Hydrogen sulfide production was evaluated using BIGGY agar, based on colour changing of colonies[31]. Overnight cultures of 80 strains were inoculated on this medium and incubated at 24ºC for 2 days. The H_2S producer strains formed brown colonies and the H_2S non-producer strains remained white. We used two starter strains as positive (YS47) and negative (YS51) control. YS 47 strains cannot produce hydrogen sulfide and YS51 starter is a low producer strain according to the industrial descriptions.

Interdelta PCR assay for separation of *Saccharomyces* strains

DNA was isolated from selected yeast strains and interdelta region was amplified using delta12 (TCAAC AATGGAATCCCAAC) (forward) and delta21 (CAT CTTAACACCGTATATGA) (reverse) primers[32]. From our collection, 35 commercially available starter strains were used as references in this experiment. PCR amplifications were carried out in 20 µL reaction volumes containing 10 µL Phusion High-Fidelity master mix (ThermoFisher Scientific, USA), 0.5 µL (10 µM) each oligonucleotide primer, 8 µL nuclease-free water and 1.0 µL DNA template. Amplification reactions were performed using the following parameters: 30 s at 98ºC followed by 35 cycles of 10 at 98ºC, 30 s at 46ºC and 1.0 min at 72ºC and a finishing step of 10 min at 72ºC. PCR products were separated on 2% agarose gel stained with ethidium bromide and visualized under UV light. The comparison of the profiles from interdelta typing was performed using the pattern analysis software GelCompar II 5.1 (Applied Maths, Belgium) using the Dice coefficient and UPGMA as clustering options[33].

Results and Discussion

Sample collection and isolation

In the present study, we report data on the biodiversity of yeast strains in the selected NARIC RIVE vineyard in Badacsonytomaj. During the isolation, we focused on the yeast strains related to the wine grape "Kéknyelű", a traditional Hungarian white wine grape variety. Samples were collected from the surface of grape berries, from fresh grape juice and from must representing different phases of wine fermentation (early, middle and final phases). Our collection also contained 35 commercial yeast strains.

Selection of isolates according to utilization of carbohydrate (CA) and nitrogen sources (NA)

Carbohydrate assimilation

The ability of 480 isolates to utilize various carbon sources was tested on agar plates using the following 11 carbohydrates as carbon source: glucose, sucrose, galactose, mannose, xylose, arabinose, maltose, glycerol, melibiose, cellobiose and rhamnose.

According to the pattern of carbohydrate assimilation, 41 groups were formed (Table 1). In general, it can be stated that all isolates, not surprisingly, utilized glucose, but the utilization of the other carbohydrates was very different and this resulted in a very large number of groups. The most utilized carbon sources were sucrose, maltose, galactose and glycerol. On the opposite, exploitation of the following three carbohydrate was under 10%: melibiose (6 cases, 1.25%), arabinose (8 cases, 1.6%) and rhamnose (38 cases, 8.3%), respectively (Fig. 1A). There were 14 groups consisting of only one isolate. On the opposite, there were 6 groups in which more than 20 isolates were classified: Group I (30 isolates), Gr. II (59 isolates), Gr. III (48 isolates), Gr. VII (63 isolates), Gr. XII (116 isolates) and Gr. XIII (36 isolates). The members of these 6 groups contain more than 73% of all isolates, presumably representing the most abundant species of the samples. There were less than 18 isolates in each of the other remaining groups. Isolates of 26 groups were able to utilize 5 or more carbohydrates, however, none of them was able to use all carbohydrate sources, but 3 isolates of groups 40 and 41 were able to utilize 10 carbohydrate sources out of 11 investigated. According to the literature, different species are capable to utilize various carbohydrate sources[34,35] but the data are controversial in some cases. Comparing our results with reference data, it turned out that they do not completely fit to each other (Suppl. Table S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*). A possible explanation for the differences could be that the utilization of carbohydrates depends on many factors like growth conditions and on the genetic background of the given strains or species.

The ITS region of some representative isolates was sequenced as described earlier. Based on the sequence analysis, 8 species (*Candida californica*, *C. zemplinina* syn. *Starmerella bacillaris*, *Hanseniaspora uvarum*, *Pichia, kluyveri*, *P. fermentans*, *Saccharomyces cerevisiae*, *S. cerevisiae/paradoxus*, *S. uvarum*) were identified. It should be mentioned that in some cases the groups harboured representatives of different genera indicating that the groups are heterogeneous.

Table 1 — Carbohydrate assimilation of yeast isolates. Results of sequence analysis (only on genus level) of randomly chosen isolates from each group are also presented

	Glucose	Sucrose	Galactose	Mannose	Xylose	Arabinose	Maltose	Glycerol	Melibiose	Cellobiose	Rhamnose	Number of isolates	Genus	Isolates CA	Isolates NA
1	+	-	-	-	-	-	-	-	-	+	-	30	*Hanseniaspora*	16	
2	+	+	-	-	-	-	-	-	-	-	-	59	*Candida*	10	
3	+	-	-	-	-	-	-	+	-	-	-	48	*Pichia*	3,4,9	
4	+	-	-	-	-	-	-	+	-	+	-	2	*ND*		
5	+	-	-	-	+	-	-	+	-	-	-	11	*Pichia*	1,2	
6	+	+	-	-	-	-	-	+	-	-	-	18	*Pichia*	5,6	
7	+	+	-	-	-	-	+	-	-	-	-	63	*Candida*, *Pichia*	7,8, 31, 32	
8	+	+	-	-	+	-	-	-	-	-	-	3	*Candida*, *Hanseniaspora*	13, 14	
9	+	+	-	-	-	+	-	-	-	-	-	1	*ND*		
10	+	+	-	-	-	-	-	-	-	-	+	1	*ND*		
11	+	+	-	-	-	-	+	+	-	-	-	5	*Pichia*	11, 12	
12	+	+	+	-	-	-	+	-	-	-	-	116	*Pichia*, *Saccharomyces*, *Hanseniaspora*,	17, 18	1-4, 6, 11-36, 38-40
13	+	+	-	-	+	-	+	-	-	-	-	36	*Candida*, *Pichia*	19, 20, 28, 35, 36	
14	+	+	-	-	-	-	-	+	-	+	-	1	*ND*		
15	+	+	-	-	-	-	-	-	-	+	+	1	*ND*		
16	+	+	+	+	+	-	-	-	-	-	-	1	*ND*		
17	+	+	+	+	-	-	+	-	-	-	-	3	*ND*		9
18	+	+	-	+	-	-	+	-	-	+	-	1	*ND*		
19	+	+	+	-	-	-	+	+	-	-	-	7	*Saccharomyces*, *Hanseniaspora*	21, 22*	5, 7, 8*,
20	+	+	-	+	-	-	+	+	-	-	-	3	*Saccharomyces*	25*, 26	10*
21	+	+	-	+	-	-	-	+	-	+	-	1	*ND*	-	
22	+	+	-	+	+	-	+	+	-	-	-	3	*Saccharomyces* *Pichia*	39, 40	
23	+	+	-	+	-	-	-	+	-	+	+	3	*ND*		
24	+	+	-	+	+	-	-	-	-	+	+	2	*ND*		
25	+	+	+	-	-	-	+	-	-	+	+	1	*ND*		
26	+	+	+	+	-	-	+	+	-	-	-	14	*Saccharomyces*	23, 24	37
27	+	+	-	+	+	-	+	+	-	+	-	1	*ND*		
28	+	+	+	+	-	-	+	+	+	-	-	1	*ND*		
29	+	+	+	-	+	-	+	+	-	+	-	6	*Pichia*	37, 38	
30	+	+	+	-	+	+	+	+	-	-	-	4	*Pichia*	29, 30	
31	+	+	+	-	-	-	+	+	-	+	+	1	*ND*		
32	+	+	-	+	-	-	+	+	-	+	+	3	*ND*		
33	+	+	-	+	+	-	-	+	-	+	+	2	*ND*		
34	+	-	+	-	+	-	+	+	+	+	-	2	*Hanseniaspora*	15	
35	+	+	+	+	+	-	+	+	-	+	-	2	*ND*		
36	+	+	+	-	+	-	+	+	-	+	+	3	*ND*		
37	+	+	+	+	+	-	+	+	-	+	+	16	*Pichia*	27, 34	
38	+	+	+	-	+	+	+	+	-	+	+	1	*Pichia*	33	
39	+	+	+	+	+	-	+	+	-	+	+	1	*ND*		
40	+	+	+	+	-	+	+	+	+	+	+	2	*ND*		
41	+	+	+	+	+	-	+	+	+	+	+	1	*ND*		
∑												480		40	40

[assimilate (+) or do not assimilate (-) carbon source, ND: not identified, *: CA22-NA8 and CA25- NA10 are the same isolates but according to the selection procedure different nomenclature were used. The identification numbers of the selected CA, NA isolates are also indicated]

Nitrogen assimilation

The method for testing the nitrogen assimilation ability of the isolates was similar to those described for utilization of carbohydrates as carbon source with the modification that instead of different carbon sources different nitrogen sources were used. Isolates were tested on agar plates supplemented with different nitrogen sources and 10 groups among the 480 isolates (Table 2) were determined based on their nitrogen assimilation ability. Three groups (Groups I, II and IV) represented 85% of all strains investigated, while in other 7 groups less than 40 isolates were identified. All 480 isolates were able to grow on minimal media supplemented with ammonium sulfate (Fig. 1B). However, none of them was able to use all nitrogen sources. In this respect, isolates of two groups (VII and X) were able to use 4 nitrogen sources out of the 5 investigated. We found that the majority of isolates were unable to assimilate sodium nitrite (99%) and potassium nitrate (93.6%). Some isolates in each group were identified by their ITS sequence and the following species were found: *Hanseniaspora uvarum*, *Pichia kluyveri*, *Saccharomyces cerevisiae*, *S. cerevisiae/paradoxus*, *S. paradoxus* and *S. uvarum*.

The results of assimilation of nitrogen sources separated most of the non-*Saccharomyces* yeasts from putative *Saccharomyces* isolates (Table 2), due to *Saccharomyces* inability to utilize lysine as a nitrogen source[36]. The 39 Lys^- and 1 Lys^+ strains (NA 1-40) were randomly selected for further analyses and molecular tests.

Similarly, 40 isolates (CA 1-40) representing the most abundant 19 groups of the carbohydrate assay (Table 1) were randomly chosen for further analyses. NA isolates were selected by focusing on the *Saccharomyces* species; while CA isolates were selected from the most abundant groups in order to

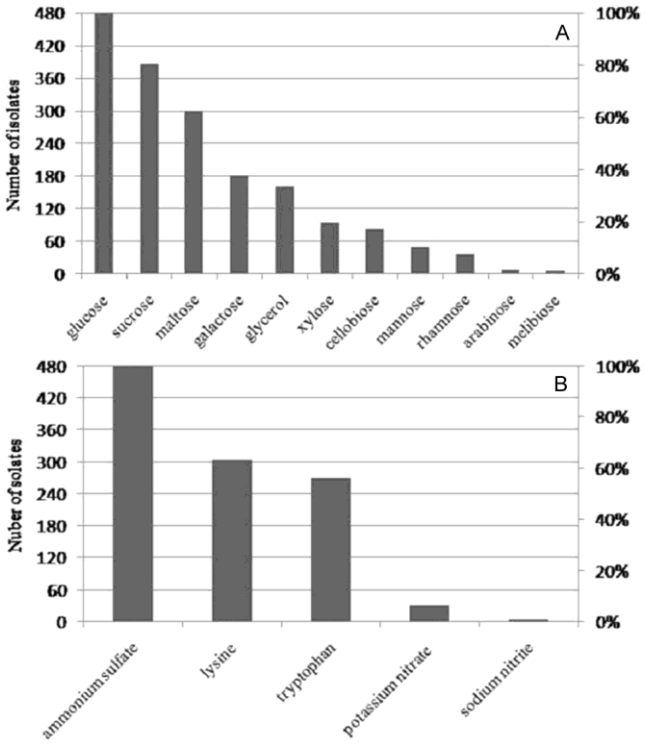

Fig. 1 — (A) Carbohydrate; and (B) Nitrogen assimilation of yeast isolates (n=480) using various carbohydrate and nitrogen sources

Table 2 — Nitrogen assimilation ability of the isolates and the results of sequence analysis (only on genus level) of randomly chosen isolates from each group

Ammonium-sulfate	Lysine	Tryptophan	Potassium-nitrate	Sodium nitrite	Number of isolates	Genus	NA isolate number
+	+	+	-	-	119	*Saccharomyces*, *Candida*	16*
+	+	-	-	-	158	*Hanseniaspora*, *Candida*	-
+	-	-	-	-	36	*Candida*, *Hanseniaspora*, *Saccharomyces*	-
+	-	+	-	-	132	*Pichia*, *Saccharomyces*	1-15, 17-40
+	-	+	+	-	1	*Candida*	-
+	-	-	+	-	6	*Candida*, *Pichia*	-
+	+	+	+	-	15	*Candida*, *Saccharomyces*	-
+	+	-	+	-	8	*Candida*	-
+	-	-	-	+	1	*Candida*	-
+	+	+	-	+	4	*Pichia*	-
					480		40

[assimilate (+); or do not assimilate nitrogen source (-); *: *Saccharomyces cerevisiae/paradoxus* is a *Saccharomyces* isolate, but it can utilize lysine]

Table 3 — Killer toxin and H_2S production of selected isolates (n=79, NA18 is missing)

Species	killer +	killer -	H_2S -	H_2S +	H_2S ++	H_2S +++	H_2S ++++
C. californica	1	0	0	1	0	0	0
C. zemplinina (*S. bacillaris*)	3	5	1	1	0	6	0
H. uvarum	2	2	4	0	0	0	0
P. fermentans	5	1	0	0	1	4	1
P. kluyveri	17	2	0	3	6	9	1
S. cerevisiae	18	12	1	23	5	1	0
S. cerevisiae/ paradoxus	6	1	0	5	2	0	0
S. uvarum	0	3	0	3	0	0	0
S. paradoxus	0	1	0	1	0	0	0

[killer +: killer toxin producer strain, killer -: killer toxin non-producer strain, H_2S -: hydrogen sulfide non-producer strain (white), H_2S +: low hydrogen sulfide producer strain (beige), H_2S ++: middle hydrogen sulfide producer strain (light brown), H_2S +++: high hydrogen sulfide producer strain (brown), H_2S ++++: very high hydrogen sulfide producer strain (dark brown)]

analyze the most representative yeast isolates. For some important characteristics of the NA and CA strains see Suppl. Tables S2 & S3.

Taxonomic identification of isolates indicates yeast diversity

Taxonomic identification of isolates was carried out by DNA sequence analysis of the PCR amplified ITS region. We were able to identify 4 groups according to their ITS fragment length. Additionally, another group was also identified consisting of isolates which showed 2 or more fragments in PCR reaction (data not shown). Randomly chosen isolates from the most abundant groups were characterized according to the sequence of the ITS region. 8 genera (*Candida*, *Cryptococcus*, *Hanseniaspora*, *Metchnikowia*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Sporidiobolus*) and the following 15 yeast species were identified: *Candida californica*, *Candida zemplinina syn. Starmerella bacillaris*, *Cryptococcus flavescens*, *Hanseniaspora uvarum*, *Metschnikowia sp.*, *Pichia fermentans*, *Pichia kluyveri*, *Rhodotorula glutinis*, *Rhodotorula nothofagi*, *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae x bayanus* (hybrid), *Saccharomyces cerevisiae/ paradoxus*, *Saccharomyces uvarum* (syn. *S. bayanus var. uvarum*), *Saccharomyces paradoxus* and *Sporidiobolus pararoseus* (Suppl. Fig. S1). Phylogenetic analysis of CA yeast strains using MEGA 7 software (Fig. 2A) showed that the CA isolates are phylogenetically separated into two clusters. Both clusters include starter yeasts and isolates presented in this work. Interestingly, the smaller cluster consists of the commercially available starter cultures with one

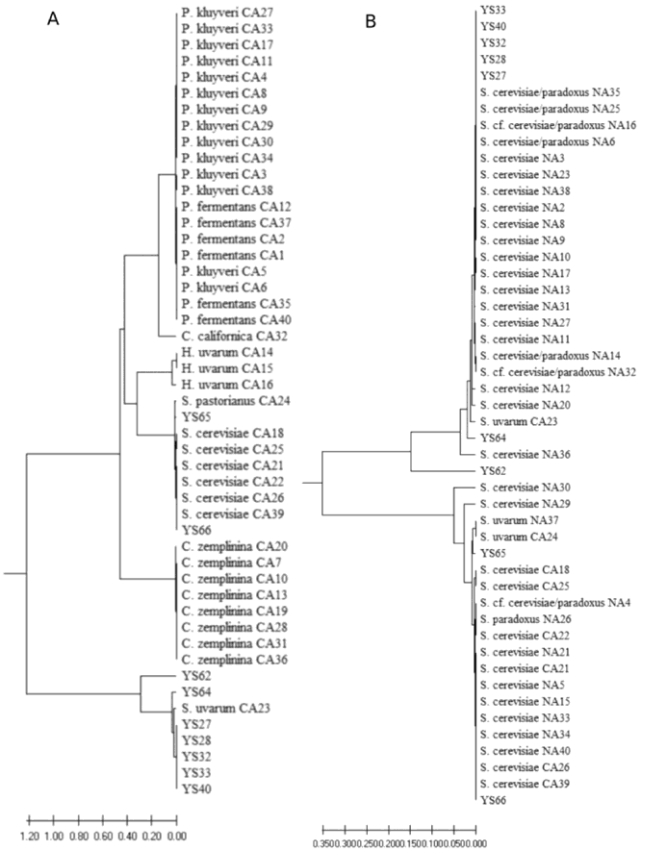

Fig. 2 — Phylogenetic tree of CA-NA isolates. (A) cluster analysis of CA1-40 and 9 starter yeasts (YS27, YS28, YS32, YS33, YS40, YS62, YS64, YS65, YS66); and (B) cluster analysis of *Saccharomyces* NA, CA strains and the same 9 commercially available starter yeast strains as indicated in part A

exception. The bigger clade is not homogenous, because it can be divided into separate subclades corresponding 4 genera (*Candida*, *Hanseniaspora*, *Pichia*, *Saccharomyces*). However, there is one exception: *C. californica* (CA32) shows more similarity to "*Pichia*" subclade than to the "*Candida*" subclade. The "*Pichia*" subclade consists of two species: *P. fermentans* and *P. kluyveri.* Similarly, the "*Saccharomyces*" subclade also consists of two species, *S. uvarum* and *S. cerevisiae*. YS65 and YS66 starter yeasts (the most frequently used starters in this winery) belong to the "*Saccharomyces*" cluster. Consequently, the phylogenetic tree of CA samples illustrates the genus and species diversity (Fig. 2A).

The phylogenetic analysis of the *Saccharomyces* species based both on NA and CA strains show that these strains can be divided into two clades (Fig. 2B), each clade can be divided into two subclades; however, only one-one strain represents a separate subclade

(YS62 and *S. cerevisiae* NA30) in this case. The strains in the two main subclades do not exhibit striking differences in the sequence of the ITS region. Therefore, it was necessary to distinguish the strains from each other using interdelta PCR analysis.

Interdelta PCR assay for separation of *Saccharomyces* strains

Delta elements can be found at the ends of Ty1 retrotransposons which are present in several copies in the yeast genome[37]. These elements are good targets for detection of polymorphism, and this technique is capable to separate different strains of the same species[38]. Different yeasts strains can produce valuable metabolites during wine fermentation, which contribute to the character of wine. In order to determine different *Saccharomyces* strains interdelta PCR analysis was performed.

Selected isolates (CA, NA) were tested with delta primers and grouped based on their specific pattern. According to the results of the analysis, 24 groups of *Saccharomyces* yeast were identified and Fig. 3 illustrates the diversity of these strains. The data obtained indicate that the *Saccharomyces* population is very diverse in the NARIC RIVE winery. Csoma *et al*. [14] also reported previously high heterogeneity of local strains from Badacsony and other wine region in Hungary. The dendrogram (Fig. 3) represents several clusters and there are only very few identical patterns observable. 24 groups were formed according to the pattern of interdelta PCR reaction. Among these 24 groups, 5 groups contain 2 isolates, one-one groups harbour 3 and 9 isolates with the same pattern (data not shown). We compared the interdelta pattern of the yeast isolates to the pattern of starter samples (data not shown) to avoid re-isolation of commercial starter yeasts. A very low number of isolates (only 3 strains) exhibited identity with the 35 starter yeasts investigated. Moreover, these starter strains do not correspond to the most frequently used starters in NARIC RIVE winery. The relative low re-isolation efficiency of the starters indicate that they could not survive successfully in the winery as they could not adapt to the local conditions. Our results are in agreement with earlier study that investigated the commercial yeast over 3 years in a winery[39]. They found that the occurrence of starter yeast was very limited in time and their colonization was not

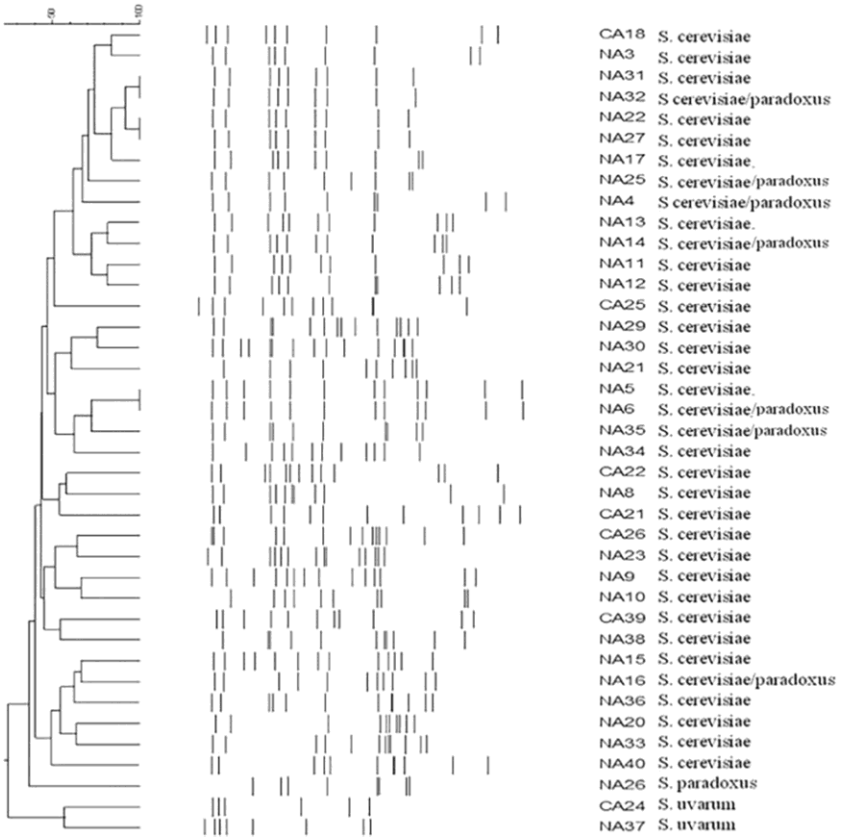

Fig.3 — Dendogram based on UPGMA cluster analysis of the *Saccharomyces* NA and CA strains

permanently observable in the environment of the studied vineyard.

Characterization of selected yeast strains

Ethanol and glucose tolerance of the isolates

Yeasts are exposed to different stress factor during fermentation like osmotic and ethanol stress. Therefore, for vinification aspect, ethanol and glucose tolerance is an important selection parameter of yeast strains. Wines usually have 10-15% ethanol concentrations and potential starter strains should tolerate these concentrations. In this study, we compared the ethanol tolerance of 80 isolates at 3 different concentrations (5-10-15%) on agar plates after 2 days of incubation. The results show that the strains did not differ significantly in the presence of 5 and 10% ethanol. Most of the isolates (76%) were not tolerant to 15% ethanol, but 96% of isolates can proliferate in 10% ethanol concentration (Fig. 4 A & C). This test turned out to be useful in order to separate potential isolates for wine making. As shown in Fig. 4A, 24% of tested strains can grow on media, which contain 15% ethanol. Isolates with 15% ethanol tolerance belong to two genera: *Saccharomyces* and *Pichia*. Similar tolerance of *Pichia* isolates compared to *Saccharomyces* strains was an unexpected result because non-*Saccharomyces* species are usually less tolerant to ethanol[1]. However, Chamnipa and colleagues[40] reported a *Pichia kudriavzevii* strain which can grow in the presence of 12% ethanol and show also high temperature tolerance as well. Ethanol tolerances of other non-*Saccharomyces* strains were investigated by Gao & Fleet[41] who found that the temperature can influence ethanol tolerance of the investigated yeasts. Their results showed *Candida stellata* and *Kloeckera apiculata* was sensitive to ethanol stress at 30ºC, but lower temperature increased the tolerance to ethanol. We also isolated some non-*Saccharomyces* strains like *P. kluyveri* or *H. uvarum* (*Kloeckera apiculata*) from Badacsony (data not shown) with higher ethanol tolerance (15 and 10% ethanol concentration), indicating that these strains can be also applied for wine fermentation.

The 80 yeast isolates were also tested for their tolerance to higher concentrations of glucose (osmotolerance) using agar plates containing 100, 200, 300 g/L glucose. Our results showed (Fig. 4 B & D) that the majority of isolates (98%) tolerated the highest glucose concentration (300 g/L) and there are not significant differences in their osmotolerance properties, which could be an important feature of strains used for wine production. The strains, which were tolerant to 15% ethanol stress, were also tolerant to glucose osmotic stress. Our results are correlation with result of Ekunsanmi & Odunfa[28], who investigated glucose tolerance of ethanol-tolerant isolates. They found that all ethanol-tolerant yeasts were able to grow at 10-25% glucose concentration.

Acid production and acetic acid tolerance

Selected 40-40 CA and NA isolates were tested on YPD agar plates complemented with $CaCO_3$. A clear zone around the yeast colony indicated acid producing activity (Fig. 5C) as observed in 25% of the isolates (Fig. 5A). Growth on YPD medium containing 1%

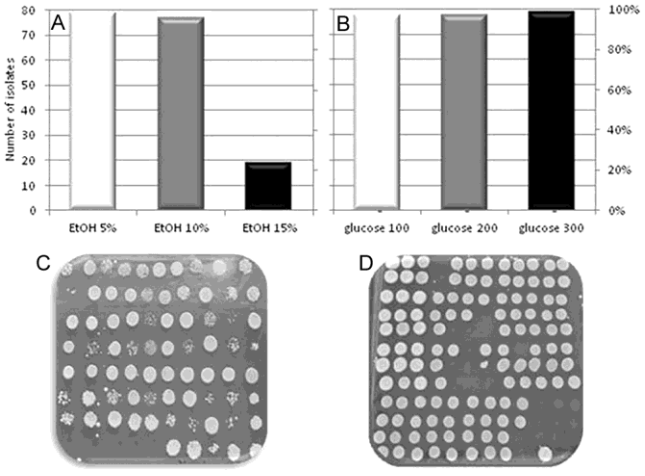

Fig. 4 — Tolerance of ethanol and glucose at 3 different concentrations (A) ethanol tolerance 5, 10, 15% EtOH; (B) glucose tolerance 100, 200, 300 g/L; (C) cell morphology of isolates on YPD agar containing 15% EtOH; and (D) cell morphology of isolates on YPD plate containing 300 g/L glucose. [Panel C and D show only representative samples]

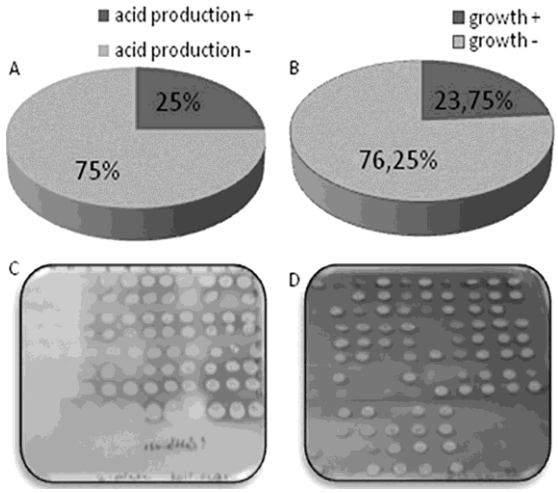

Fig. 5 — Acid production and acetic acid tolerance of isolates. (A and B) acid production and tolerance (%), respectively; (C) clear zone around the colonies on $CaCO_3$ containing agar plate indicating acid production; and (D) growth and cell morphology of isolates on 1% acetic acid containing agar

acetic acid was indicative for acid tolerance (Fig. 5D), which was detected in 23.75% of yeast isolates (Fig. 5B). All of the strains, which were tolerant to acetic acid stress, also produced acid. We found one strain that synthesized acid but showed intolerance for acetic acid in this test.

Fermentation ability

The glucose fermentation ability was also evaluated for the 80 CA and NA strains. As a positive control, glucose rich medium, and as a negative control glucose-free medium was used and both of them were inoculated with the same commercially available yeast starter (YS47). Samples were incubated at three different temperatures viz. 26, 21 and 16ºC. The Though the temperature of 25-26ºC is optimal for the growth of yeast cells, the industrial wineries use lower temperatures such as 16-18ºC for fresh and aromatic wine production.

In this test, fermentation ability of 80 NA and CA strains representing 9 different species (*Candida califronica*, *Candida zemplinina (syn. Starmerella bacillaris)*, *Hanseniaspora uvarum*, *Pichia fermentans*, *Pichia kluyveri*, *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae/paradoxus*, *Saccharomyces uvarum*, *Saccharomyces paradoxus*) and 3 commercial strains were analyzed. Fig. 6 shows the fermentation profile of representative nine yeasts including starter yeast (YS47). The most yeast strains which were determined as *S. cerevisiae*, started fermentation at the first or second day at 26ºC and third day at 21 and 16ºC, except for two isolates (NA15 and CA22). Isolate NA15 was capable fermenting at only 16ºC in contrast; CA22 could not ferment at 16ºC but at the other two tested temperatures only (data not shown). We found two *H. uvarum* strains, which started to ferment glucose on the first day at 26ºC but decreasing the temperature to 16ºC caused a 1-day shift in beginning of fermentation. The tested 19 *P. kluyveri* strains showed different fermentation abilities, 6 isolates fermented slow (start on 5th day only) at 26 and 21ºC and they could not ferment at 16ºC, except one strain (CA35). Other *P. kluyveri* strains started fermentation after 2 or 3 days at 26ºC and only one strain (CA27) could ferment on third day at 16ºC, other strains just later. The *C. californica* strain (CA32) could not perform fermentation at 16 and 26ºC, but showed fermentation only at 21ºC after 6 days. We noticed differences in the fermentation ability of *C. zemplinina* (syn. *Starmerella bacillaris*) strains at 16ºC. The fermentation started from days 3 to 6 and in some cases complete fermentation was not observable. The *C. zemplinina* isolate (CA28) started to ferment glucose on day 1 at 26 and 21ºC, and on the third day it fermented glucose at 16ºC. Most isolates reached the maximum capacity after 6 days at all temperatures,

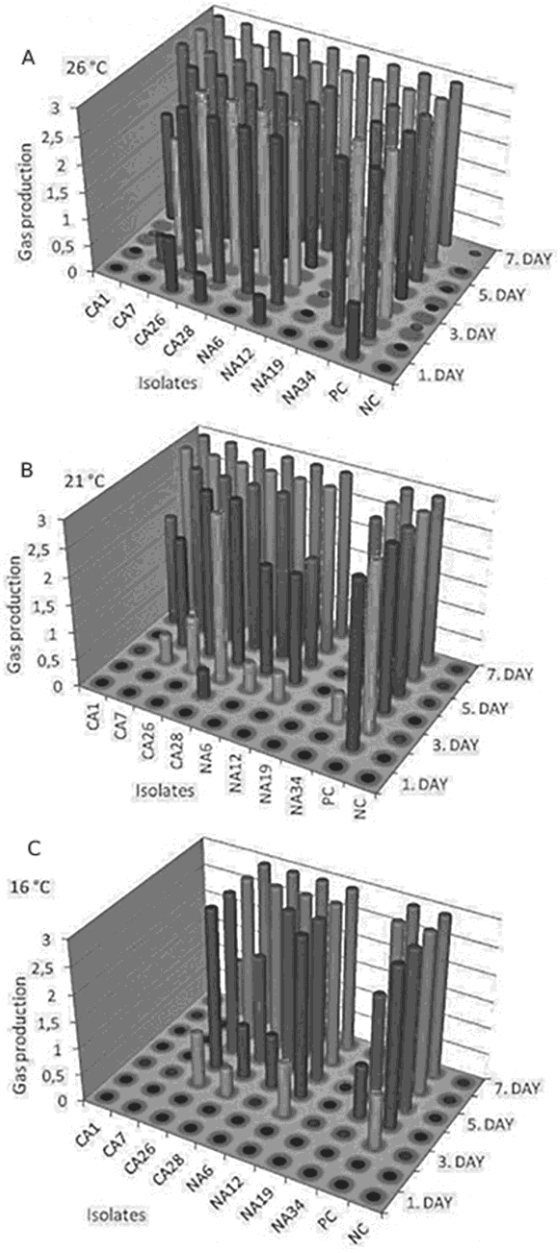

Fig. 6 — Glucose fermentation rates of 8 isolates in 7 days, at 3 different temperatures, isolates: CA1 (*P. fermentans*), CA7 (*C. zemplinina* syn. *S. bacillaris*), CA26 (*S. cerevisiae*), CA28 (*C. zemplinina* syn. *S. bacillaris*), NA6 (*S. cf. cerevisiae/ paradoxus*), NA12 (*S. cerevisiae*), NA19 (*P. kluyveri*), NA34 (*S. cerevisiae*); PC: positive control, YS47, inoculated with starter yeast; and NC: negative control, glucose free medium inoculated with same starter yeast (YS47), A-C: fermentation intensity at 26, 21 and 16ºC, respectively

except those strains which could not ferment at all. TMost of the strains showed fermentation better at 26ºC than 21 or 16ºC (Fig. 6). In general, decreasing the temperature, the fermentation intensity also decreased. According to our results, 10 samples (CA16, CA26, NA5, NA12, NA13, NA15, NA25, NA27 NA36 and NA37) among the studied isolates showed same fermentation ability as YS47 starter strain. Furthermore, CA19 and NA14 strains performed faster fermentation than the positive control strain at 16ºC. These strains have potential to be used as local strains in wine making, but it is necessary to investigate other characteristics as well.

Killer toxin and hydrogen sulfide production

Killer yeasts strains can produce toxin against sensitive yeasts to kill them and as a consequence decreasing the number of competitor yeasts. In wine making, non-sterile must are used for fermentation, therefore, many microorganisms are present in the must. These microorganisms are in competition with each other for available substrates, so it is beneficial in wine making that the selected starter strains have killer activity. In literature, there are many yeasts species with killer character, like *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Metschnikowia pullcherrima*, *Pichia kluyveri*, etc[42,43]. We analyzed killer toxin production of our selected isolates and the results are shown in Table 3. Among the 80 isolates, 52 killer positive strains were determined belonging to 7 species. Most of strains showed killer activity was *Pichia kluyveri* and *Saccharomyces cerevisiae* species. The fact that relative high number of killer yeasts strains (65%) were detected among the investigated yeasts isolated from fermented must samples, might reflect an environmental enrichment effect. Our results are in agreement with previously studies, regarding differences of *Pichia guilliermondii*[43] and *Wickerhamomyces anomala*[44] in their killer sensitivity depending on the environmental origin of isolates. According to their results, the strains isolated from fermented must were mostly resistant to killer toxin in contrast to strains isolated from grapes. This observation can be explained by the toxin production of some killer strains present in the must, thus toxins eliminate the sensitive competitors. Therefore, the surviving yeasts were adapted to the killer toxin-reach environment in the must, and as a consequence, the number of the killer toxin resistance strains emerged.

Hydrogen sulfide production of yeasts is an undesirable feature in winemaking affecting the foul taste and smell of the final product. In our experiments the positive control starter yeast strains also produced hydrogen sulfide at low level (YS47), so we regarded such isolates, which showed the same hydrogen sulfide production level, as acceptable strains for wine making. Production of hydrogen sulfide was tested on BIGGY agar based on colour changing (white colony: non-producer strain, beige colony: low producer strain, light brown colony: middle producer strain, brown colony: high producer strain, dark brown colony: very high producer strain).

As shown in Table 3, 74 isolates produced hydrogen sulfide among 80 isolates. The non-producer strains (CA14, CA15, CA16, NA7, CA28, and NA2) belong to *H. uvarum* (4), *C. zemplinina* syn. *Starmerella bacillaris* (1) and *S. cerevisiae* (1) species. If we are looking for low level hydrogen sulfide producer strains, we can identify 37 isolates including *Saccharomyces* strains in high number. We found 43 strains (non-producer and low producer) which could be adaptable for wine fermentation based on hydrogen sulfide production.

High number of yeast strains was isolated from the "Badacsony" region in Hungary, where local wine yeast population was not investigated in detail, except one publication[14]. Eight genera and 15 yeast species were identified from 480 isolates from grape and must samples derived from NARIC RIVE viticulture. All of them were characterized by carbohydrate and nitrogen utilization assay (Tables 1 & 2). We identified differences of carbohydrate utilization of some species compared to literature data and these results were presented in Suppl. Table S1. Our results have demonstrated that the carbon and nitrogen assimilation tests were effective for selecting isolates for further investigations. The nitrogen assimilation test was successful in screening *Saccharomyces* isolates after confirmation by ITS sequence analysis. All of these observations suggest that both microbiological and molecular methods were useful in isolation and characterization of local microbes. The comparison of microbiological and molecular biological data strengthened this observation. According to the results of interdelta PCR analysis (Fig. 3), the *Saccharomyces* yeast community of the NARIC RIVE winery is very diverse, as 24 out of 39 *Saccharomyces* isolates were identified as different ones. The fact that the isolated strains were collected from one winery, moreover, from one type of grape and must, underlines once more the above statement.

We also analyzed commercially available starter yeast strains and compared them to our isolates to avoid re-isolation; furthermore, they were also used as reference strains. We were able to isolate starters from grape and must samples only in a few cases despite their intensive use in this winery. Moreover, the re-isolated starters do not represent the most frequently used commercial starters. The relatively low incidence of commercial starters and the dominance of "terroir" yeasts indicate that presumably the local microbiota is better adapted to the natural conditions and fermentation processes.

Analysis of the following physiological and oenological characters of isolates: ethanol and glucose tolerance (Fig. 4), acid production and tolerance (Fig. 5), fermentation ability (Fig. 6), killer toxin and hydrogen sulfide production (Suppl. Tables S2 & S3) were used for screening of potentially beneficial strains for winemaking. Observation of several oenological features together allowed selecting the most suitable strains for fermentation. We found 36 proper strains (underlined in Suppl. Tables S2 & S3) from local yeasts population according to their character of glucose tolerance (300 g/L), ethanol tolerance (10 and 15%) and hydrogen sulfide production (non- and low producer strains). Among the 36 strains we could select some wild yeast strains that showed even better technological properties, than the control starter used, like CA19 and NA14 strains. These two strains showed faster fermentation ability at 16ºC than the starter strains studied. This estimation strengthens the observation that the isolated and selected strains could be applied in wine production and can contribute to assure the particular wine characteristics of the Badacsony region.

Conclusion

In this study, we explored the biodiversity of the yeast community in a Badacsony winery and designed an optimal method for selection of potential starter yeasts from the diverse local microbiome. We isolated 480 local yeast strains from the Badacsony wine region in Hungary and characterized on the basis of their ability to be used as "terroir" starter cultures in wine fermentation. Carbohydrate and nitrogen utilization studies significantly supported effective selection of isolates for further studies. Parallel application of microbiological and molecular methods was useful in characterizing local microbes. The *Saccharomyces* population was diverse though isolated from a single type of grape and must. The dominance of "terroir" yeasts over commercially available starter cultures used in winemaking suggests that the local microbiota is better adapted to natural conditions. Analysis of the physiological and oenological properties of the isolates showed that most of the *Saccharomyces* isolates can be used as starter cultures in wine fermentation.

Conflict of interest

Authors declare no conflict of interests.

References

1 Fleet GH, Wine Yeasts for the Future. *FEMS Yeast Res*, 8 (2008) 979.

2 Mills DA, Johannsen EA & Cocolin L, Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl Environ Microbiol*, 68 (2002) 4884.

3 Capece A, Romaniello R, Pietrafesa R & Romano P, Indigenous *Saccharomyces cerevisiae* yeasts as a source of biodiversity for the selection of starters for specific fermentations. *BIO Web Conf*, 3 (2014) 02003.

4 Viana F, Gil JV, Genovés S, Vallés S & Manzanares P, Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiol*, 25 (2008) 778.

5 Fleet GH, Yeast interactions and wine flavour. *Int J Food Microbiol*, 86 (2003) 11.

6 Arora B & More S, Impact of pectolytic enzyme on the fermentation time and free-run juice rate of Sharad red wine. *Indian J Exp Biol*, 53 (2015) 104.

7 Fernández M, Ubeda JF & Briones AI, Typing of non-Saccharomyces yeasts with enzymatic activities of interest in wine-making. *Int J Food Microbiol*, 59 (2000) 29.

8 Fernández-González M, Ubeda JF, Vasudevan TG, Cordero Otero RR & Briones AI, Evaluation of polygalacturonase activity in Saccharomyces cerevisiae wine strains. *FEMS Microbiol Lett*, 237 (2004) 261.

9 Suzzi G, Arfelli G, Schirone M, Corsetti A, Perpetuini G & Tofalo R, Effect of grape indigenous *Saccharomyces cerevisiae* strains on Montepulciano d'Abruzzo red wine quality. *Food Res Int*, 46 (2012) 22.

10 Binati LR, Innocente G, Gatto V, Celebrin A, Polo M, Felis GE & Torriani S, Exploring the diversity of a collection of native non-Saccharomyces yeasts to develop co-starter cultures for winemaking. *Food Res Int*, 122 (2019) 432.

11 Rainieri S & Pretorius IS, Selection and improvement of wine yeasts. *Ann Microbiol*, 50 (2000) 15.

12 Guimarães TM, Moriel DG, Machado IP, Picheth CMTF & Bonfim TMB, Isolation and characterization of *Saccharomyces cerevisiae* strains of winery interest. *Braz J Pharm Sci*, 42 (2006) 119.

13 Regodón JA, Peréz F, Valdés ME, De Miguel C & Ramírez M, A simple and effective procedure for selection of wine yeast strains. *Food Microbiol*, 14 (1997) 247.

14 Csoma H, Zakany N, Capece A, Romano P & Sipiczki M, Biological diversity of *Saccharomyces* yeasts of spontaneously fermenting wines in four wine regions: Comparative genotypic and phenotypic analysis. *Int J Food Microbiol*, 140 (2010) 239.

15 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*, 28 (2011) 873.
16 Kapsopoulou K, Mourtzini A, Anthoulas M & Nerantzis E, Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae. World J Microbiol Biotechnol*, 23 (2007) 735.
17 Dutraive O, Benito S, Fritsch S, Beisert B, Patz CD & Rauhut D, Effect of Sequential Inoculation with Non-Saccharomyces and Saccharomyces Yeasts on Riesling Wine Chemical Composition. *Fermentation*, 5 (2019) 79.
18 Toro ME & Vazquez F, Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine yeasts. *World J Microbiol Biotechnol*, 18 (2002) 347.
19 Escribano-Viana R, González-Arenzana L, Portu J, Garijo P, López-Alfaro I, López R, Santamaría P & Gutiérrez AR, Wine Aroma Evolution throughout Alcoholic Fermentation Sequentially Inoculated with Non-Saccharomyces/ Saccharomyces Yeasts. *Food Res Int*, 112 (2018) 17.
20 Binati RL, Lemos Junior WJF, Luzzini G, Slaghenaufi D, Ugliano M & Torriani S, Contribution of Non-Saccharomyces Yeasts to Wine Volatile and Sensory Diversity: A Study on Lachancea thermotolerans, Metschnikowia Spp. and Starmerella bacillaris Strains Isolated in Italy. *Int J Food Microbiol*, 318 (2020) 108470.
21 Lopes CA, Sa´ ez JS & Sangorrı´n MP, Differential response of *Pichia guilliermondii* spoilage isolates to biological and physicochemical factors prevailing in Patagonian wine fermentations. *Can J Microbiol*, 55 (2009) 801.
22 Baffi MA, Dos Santos Bezerra C, Arévalo-Villena M, Briones-Pérez AI, Gomes E & Da Silva R, Isolation and molecular identification of wine yeasts from a Brazilian vineyard. *Ann Microbiol*, 61 (2011) 75.
23 Kurtzman CP, Fell JW, Boekhout T & Robert V, Methods for isolation, phenotypic characterization and maintenance of yeasts. In: *The Yeasts*, 5th Edn., (Eds. Kurtzman C, Fell JW,& Boekhout T; Elsevier, Amsterdam), 2011.
24 White TJ, Bruns T, Lee S & Taylor JW, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*, (Eds. Innis MA, Gelfand DH, Sninsky JJ & White TJ; Academic Press, New York), 1990.
25 Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ, Basic local alignment search tool. *J Mol Biol*, 215 (1990) 403.
26 Kumar S, Stecher G & Tamura K, MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol*, 33 (2016) 1870.
27 Lee YJ, Choi YR, Lee SY, Park JT, Shim JH, Park KH & Kim JW, Screening Wild Yeast Strains for Alcohol Fermentation from Various Fruits. *Mycobiology*, 39 (2011) 33.
28 Ekunsanmi TJ & Odunfa SA, Ethanol tolerance, sugar tolerance and invertase activities of some yeast strains isolated from steep water of fermenting cassava tubers. *J Applied Bacteriol*, 69 (1990) 672.
29 Jimoh SO, Ado SA, Ameh JB & Whong CMZ, Osmotolerance and Fermentative Pattern of Brewer ' s Yeast. *Word J Life Sci Med Res*, 2 (2012) 59.
30 Izgu F, Altinbay D & Yucelis A, Identification and killer activity of a yeast contaminating starter cultures of *Saccharomyces cerevisiae* strains used in the Turkish baking industry. *Food Microbiol*, 14 (1997) 125.
31 Mauriello G, Capece A, D'Auria M, Garde-Cerdán T & Romano P, SPME-GC method as a tool to differentiate VOC profiles in *Saccharomyces cerevisiae* wine yeasts. *Food Microbiol*, 26 (2009) 246.
32 Legras JL & Karst F, Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. *FEMS Microbiol Lett*, 221 (2003) 249.
33 Gürtler V, Harford C, Bywater J & Mayall BC, Direct identification of slowly growing *Mycobacterium* species by analysis of the intergenic 16S-23S rDNA spacer region (ISR) using a GelCompar II database containing sequence based optimization for restriction fragment site polymorphisms (RFLPs) for 12 enzymes. *J Microbiol Methods*, 64 (2006) 185.
34 Rib'ereau-Gayon P, Dubourdieu D, Don`eche B & Lonvaud A, Classification of yeast species, In: *Handbook of Enology Vol. 1 The Microbiology of Wine and Vinifications* 2nd Edn. (Eds. Rib'ereau-Gayon P, Dubourdieu D, Don`eche B & Lonvaud A; John Wiley & Sons, West Sussex, England), 2006.
35 Vaughan-Martini A & Martini A, Saccharomyces Meyen ex Reess (1870), In: *The Yeasts 5th Edition* (Ed. Kurtzman C, Fell JW & Boekhout T, Elsevier, Amsterdam), 2011.
36 Walters BLS, Thiselton MR, Schultz AS & Zealand N, Utilization of lysine by yeasts. *J Inst Brewing*, 59 (1953) 401.
37 Guillamón JM & Barrio E, Genetic Polymorphism in Wine Yeasts: Mechanisms and Methods for Its Detection. *Front Microbiol*, 8 (2017) 806.
38 Xufre A, Albergaria H, Gírio F & Spencer-Martins I, Use of interdelta polymorphisms of *Saccharomyces cerevisiae* strains to monitor population evolution during wine fermentation. *J Ind Microbiol Biotechnol*, 38 (2011) 12.
39 Cordero-Bueso G, Arroyo T, Serrano A & Valero E, Remanence and survival of commercial yeast in different ecological niches of the vineyard. *FEMS Microbol Ecol*, 77 (2011) 429.
40 Chamnipa N, Thanonkeoc S, Klanritb P & Thanonkeo P, The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8-1 for high-temperature ethanol production. *Braz J Microbiol*, 49 (2018) 378.
41 Gao C & Fleet GH, The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata. J Applied Bacteriol*, 65 (1988) 405.
42 Antunes J & Aguiar C, Search for killer phenotypes with potential for biological control. *Ann Microbiol*, 62 (2011) 427.
43 Lopes CA & Sangorrín MP, Optimization of killer assays for yeast selection protocols. *Rev Argent Microbiol* 42 (2010) 298.
44 Loureiroand V & Malfeito-Ferreira M, Detecting spoilage yeasts. In: *Understanding and Measuring the Shelf-life of Food*, (Ed. Steele R, Woodhead Publishing Limited, Cambridge, England), 2004.