Characterization and selection of *Saccharomyces cerevisiae* strains isolated from traditional and newly-bred vine varieties of Czech Republic and Slovakia

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Summary

Controlled fermentation with a pure *Saccharomyces cerevisiae* culture is a frequent practice in modern wineries. Use of other fermentation strategies could positively influence the aroma of the wine product. However, the background and application of these approaches are often very difficult and complex. The use of axenic *S. cerevisiae* culture originating from the particular terroir remains the best compromise for modern winemaking. In our research, 48 *S. cerevisiae* strains from 7 viticultural regions of Czech Republic and Slovakia were isolated, identified and studied. For the first time, newly-bred vine varieties Devín, Pálava, Moravian Muscat and Dunaj were used for isolation of new yeast strains. The aim of this work was to find a convenient method for identification and selection of autochthonous *S. cerevisiae* yeasts. Isolated strains were identified using conventional microbiological tests, sequencing of the internal transcribed spacer region and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). From the results of this research, the selection of 17 yeast strains for potential use as a starter culture in the production of typical wines was performed. Six of them, namely, PAC34, PAE58, PAG63, DUH135, PAH48 and DEH53, were isolated from newly-bred varieties.

Keywords

autochthonous yeast; *Saccharomyces*; isolation; selection; wine

Wine quality is strongly influenced by the yeast strain used for fermentation. The influence of various *Saccharomyces cerevisiae* strains on sensory properties of wine was described [1, 2]. At the time when application of commercial pure cultures of wine yeasts is a common oenological practice of modern winemaking, some producers are still using traditional spontaneous fermentation with autochthonous yeasts. The latter yeasts, both *Saccharomyces* and non-*Saccharomyces* ones, are the part of natural microbiome covering habitus of vine and very well adapted to conditions in a particular environment. Depending on their specific properties and abilities, they can process the fermentation and influence the final wine both positively and negatively.

A survey of spontaneous fermentation in European viticultural regions showed some particularities of indigenous *Saccharomyces* yeasts and the dominance of several strains of *S. cerevisiae* during fermentation. The commercial strains are commonly used as starters to control the quality of vinification and to prevent spoilage of wine. The other possibility is to use autochthonous strains isolated from natural sources. Selection of autochthonous yeasts would increase diversity in winemaking, improving the wine complexity and ensuring regional typicity of wines [3–5].

The Slovakia and Czech Republic are young sovereign countries, both founded in 1993 after the peaceful dissolution of Czechoslovakia. Czech and Slovak winemakers mostly produce

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fresh monovarietal wines made by technology of controlled fermentation with commercial yeast strains. Before the Velvet revolution, winemakers focused on high yields of grapes, which was in conflict with high quality of wines. On the positive side, a network of research stations was built. The first active dry yeast product in Central Europe called "VIAKVAS 82" was produced in 1982 in Czechoslovakia [6]. As a result of the researchers' efforts, new varieties of *Vitis vinifera* were bred and are used until today. In this research, some newly-bred varieties were used for isolation of wine yeasts (Tab. 1 [7]). These new varieties were bred to achieve high sugar concentration of grapes in regional climatic conditions. Although the yeast industry offers a miscellaneous variety of strains suitable for different types of vinifications as well as for specific vine varieties, strains for newly-bred varieties have not been isolated and characterized yet.

The aim of this study was to isolate autochthonous *S. cerevisiae* strains suitable for application in winemaking, especially in vinification of newly-bred grape varieties. To identify the isolates, three ways of identification were used: conventional methods, sequencing of the internal transcribed spacer ITS1-5.8S rDNA-ITS2 region and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). Results of these methods were compared. To characterize basic oenological properties, ethanoltolerance and osmotolerance, tendency of hydrogen sulphide production, β-glucosidase activity, growth in liquid medium, fermentation rate and volatile acids production were determined.

Tab. 1. Newly-bred vine varieties from Czech Republic and Slovakia [7].

Vine variety	Parental varieties	Registration year
Devín	Rot Traminer × Frühroter Veltliner	1997/1998*
Moravian Muscat	Muscat Ottonel × Prachtraube	1987
Pálava	Rot Traminer × Müller-Thurgau	1977
Dunaj	(Muscat Bouchet × Oporto) × Saint Laurent	1997

Varieties Devín and Dunaj were bred at the Research Institute of Viticulture and Enology (Bratislava, Slovakia) in 1958 by D. Pospíšilová and O. Korpás.
Variety Moravian Muscat was bred at Polešovice (Moravia, Czech Republic) by V. Křivánek.
Variety Pálava was bred in Perná (Moravia, Czech Republic) in 1950s by J. Veverka.
* – registered in 1997 in Slovakia and in 1998 in Czech Republic.

MATERIALS AND METHODS

Grape samples

Grape samples were collected in several viticultural regions of Czech Republic and Slovakia (Fig. 1). In addition to traditional vine varieties, some of the newly-bred varieties such as Devín, Pálava, Moravian Muscat (aromatic white varieties) and Dunaj (red variety) were used (Tab. 2). Approximately 500 g of well matured, healthy and undamaged grapes of each sample were aseptically placed into sterile plastic bags a few days before the harvest.

Yeasts isolation

Samples of grapes were aseptically crushed and the separated grape juice was fermented in 250 ml sterile flasks with air locks. In order to inhibit bacterial contamination, 100 $mg \cdot l^{-1}$ of chloramphenicol (Carl Roth, Karlsruhe, Germany) was added. Samples were incubated for 12–20 days at laboratory temperature. In several periods (after 2, 3, 4, 6, 8, 12 and 20 days), 300 μl of the fermenting must was sampled and inoculated, in duplicate, on yeast extract-dextrose agar (YDA; yeast extract 10 $g \cdot l^{-1}$, glucose 20 $g \cdot l^{-1}$, agar 15 $g \cdot l^{-1}$, pH 6.5) with the addition of chloramphenicol (100 $mg \cdot l^{-1}$) and incubated at 28 °C for 48 h. Approximately 20 yeast colonies were isolated from each sampling. A total of 1 634 yeast colonies were isolated and investigated by light microscope Leica DM 1000 (Leica Microsystems, Nussloch, Germany). Based on colony morphology and morphology of yeast cells examined by light microscopy, 231 isolates were selected. These strains were purified using an inverted light microscope Leica DMi8 (Leica Microsystems) with a motor-driven micromanipulator Narishige MM-92 (Narishige, Tokyo, Japan) and pneumatic microinjector Narishige IM-9B (Narishige). A single yeast cell was placed into 2 ml of liquid yeast extract-dextrose (YD) medium (yeast extract 10 $g \cdot l^{-1}$, glucose 20 $g \cdot l^{-1}$) with 100 $mg \cdot l^{-1}$ of chloramphenicol, pH 6.5. After 24 h of incubation at 28 °C, axenic cultures of yeasts were obtained. Pure cultures were stored in 50% (v/v) glycerol stocks at –80 °C for log term storage or on YDA slants at 4 °C for short term storage.

Yeasts identification by conventional methods

Conventional identification of yeasts was carried out following the taxonomic criteria [8]. To avoid unnecessary testing, only the discriminating tests for wine yeasts, based on their morphological, sexual, physiological and biochemical characteristics were carried out [9]. Cell morphology and vegetative reproduction were examined after 48 h

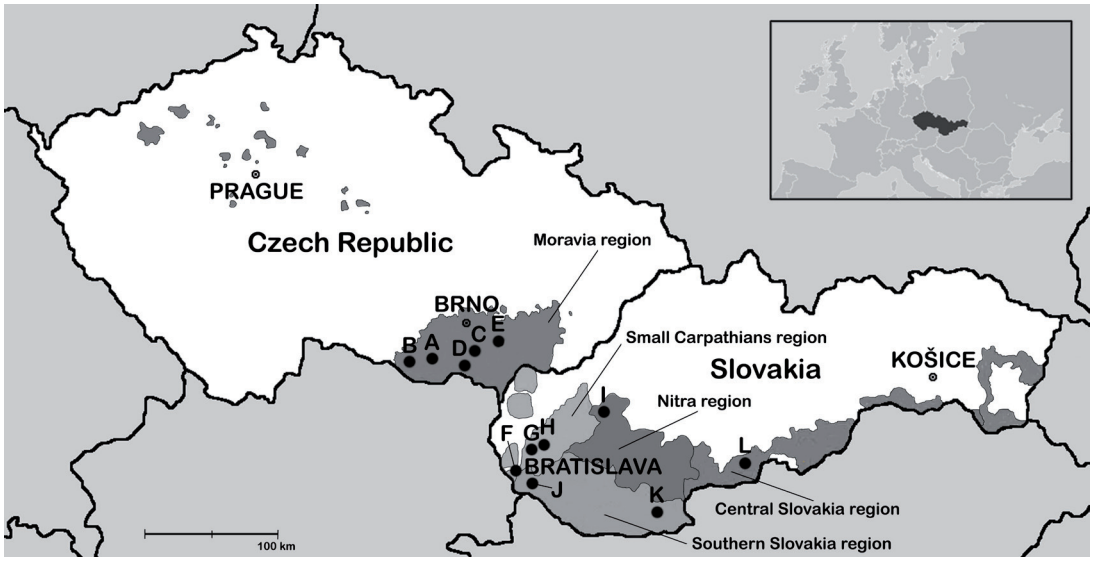

Fig. 1. Map of Czech Republic and Slovakia showing locations of sampling sites.

Samples were taken in 5 different areas of Czech Republic: Stošíkovice (A), Dobšice (B), Popice (C), Březí (D) and Krumvíř (E) and in 7 areas of Slovakia: Bratislava (F), Pezinok (G), Modra (H), Radošina (I), Šamorín (J), Strekov (K) and Modrý Kameň (L). Sampling sites are listed in Tab. 2.

Tab. 2. Origin of grape samples.

Country	Viticultural Region/Subregion	Viticultural Area	Vine Variety	Sample ID
Czech Republic	Moravia/Znojmo	Stošíkovice (A)	Riesling	RRA
		Dobšice (B)	Riesling	RRB
	Moravia/Mikulov	Popice (C)	Sauvignon Blanc	SBC
			Riesling	RRC
			Pálava	PAC
		Březí (D)	Welschriesling	WRD
	Moravia/Velké Pavlovice	Krumvíř (E)	Pálava	PAE
			Riesling	RRE
Slovakia	Small Carpathians	Bratislava (F)	Blaufränkisch	BFF
		Pezinok (G)	Pálava	PAG
		Modra (H)	Dunaj	DUH
			Welschriesling	WRH
			Moravian Muscat	MMH
			Pálava	PAH
			Devín	DEH
	Nitra	Radošina (I)	Pinot Gris	PGI
			Pinot Noir	PNI
	Southern Slovakia	Šamorín (J)	Müller-Thurgau	MTJ
		Strekov (K)	Riesling	RRK
	Central Slovakia	Modrý Kameň (L)	Gewürztraminer	GTL

Sample ID was created from the letters representing vine variety (RR – Riesling, SB – Sauvignon blanc, PA – Pálava, WR – Welschriesling, BF – Blaufränkisch, DU – Dunaj, MM – Moravian Muscat, PG – Pinot gris, PN – Pinot noir, MT – Müller-Thurgau, GT – Gewürztraminer) and area of origin (A–L) marked in Fig. 1.

of incubation in liquid YD medium at laboratory temperature. Sexual reproduction was induced by 7 days of incubation on sodium acetate agar (Merck, Darmstadt, Germany) at laboratory temperature [10]. Utilization of carbon sources was tested by the auxanographic method on yeast nitrogen base medium (Difco Laboratories, Detroit, Michigan, USA) and utilization of nitrogen sources was tested on yeast carbon base medium (Difco Laboratories) after 7 days at laboratory temperature. Fermentation of various sugars (D-glucose, D-galactose, saccharose, maltose, lactose, raffinose α–α-trehalose and melezitose) was used for routine identification. The sugars were tested

as 20 $g \cdot l^{-1}$ solutions in liquid basic medium (yeast extract 10 $g \cdot l^{-1}$, bromothymol blue 20 $mg \cdot l^{-1}$) in tubes, which contained small inverted tubes (Durham tubes). The tubes were incubated at laboratory temperature for up to 28 days [8]. Growth at 37 °C and 40 °C was assessed on solid YDA medium. Slants were inoculated with cells of a young culture and incubated at each temperature for 4 days and then inspected for growth. Vitamin requirement test was carried out on vitamin-free yeast base medium (Difco Laboratories) for 7 days at laboratory temperature [8]. In total, 48 strains were identified as *Saccharomyces cerevisiae*.

Screening of oenological properties

Technological characteristics as growth in liquid medium, volatile acids production, production of hydrogen sulphide and β-glucosidase activity defined by Resolution OIV-OENO 370-2012 [11] were investigated. All tests were carried out in triplicate using a 24 h yeast culture prepared in YD medium, in aerobic conditions on an orbital shaker at 2 Hz, 28 °C, with a starting cell concentration of approximately 10^5 $CFU \cdot ml^{-1}$.

Osmotolerance was tested as a combination of tolerance to the high concentrations of ethanol and glucose. Ethanoltolerance was tested in 5 ml of YD medium supplemented with 12.5, 15, 17.5, 20 and 22.5% (v/v) ethanol (96% ethanol for UV, Centralchem, Bratislava, Slovakia). Ethanol was added after the sterilization. Osmotolerance was tested in test-tubes with 5 ml of YD medium with increased concentration of glucose (30, 40, 45, 50, 55 and 60% (w/w)). Inoculated tubes were incubated for 10 days at 28 °C. Viability of yeast cells was evaluated by light microscopy.

Production of hydrogen sulphide (H_2S) was tested by inoculating the yeasts on BiGGY agar (Oxoid, Basingstoke, United Kingdom). Yeasts were incubated at 28 °C for 2 days and the colour of yeast colonies was evaluated as follows: 0 (white colonies, no hydrogen sulphide production), 1 (cream colonies), 2 (light brown colonies), 3 (brown colonies), 4 (dark brown or black colonies, very intensive hydrogen sulphide production).

Investigated strains were characterized for their β-glucosidase (EC 3.2.1.21) activity using a medium containing esculin (esculin 1 $g \cdot l^{-1}$, ferric ammonium citrate 0.5 $g \cdot l^{-1}$, yeast extract 10 $g \cdot l^{-1}$, agar 15 $g \cdot l^{-1}$) [12]. To assay the enzymatic activity semi-quantitatively, the strains were streaked onto plates and incubated at 28 °C for 48–72 h. Strains with β-glucosidase activity hydrolyse the substrate in medium and the dark brown zone develops around the colonies. The diameter of the brown halo around the colonies was measured in millimetres [13]. Results were expressed as 0 (no activity), 1 (weak activity; 0–5 mm), 2 (moderate activity; 5–10 mm) and 3 (strong activity; ≥10 mm).

The mode of growth in liquid medium of yeasts during fermentation is an important technological characteristic as it can affect the management of fermentative process. After incubation in liquid YD medium at laboratory temperature for 3 days, production and appearance of sediment and surface structures were monitored.

Furthermore, the isolates were tested for other oenological properties, namely, fermentation rate and volatile acids production. Microfermentation trials were carried out in 400 ml of a highly nutritive medium (yeast extract 10 $g \cdot l^{-1}$, glucose 240 $g \cdot l^{-1}$, pH 3.5) at a temperature of 21 °C [14]. All fermentations started with a concentration of yeast cells of 10^6 $CFU \cdot ml^{-1}$. The fermentation activity was followed by daily monitoring of CO_2 production. Fermentations were stopped when the weight remained stable for two consecutive days. Afterwards, samples of fermented media were analysed. Basic technological parameters as well as basic volatile profiles were measured. Basic chemical parameters of all samples were determined by methods of the International Organization of Vine and Wine (OIV) [15]. Fermentation rate was expressed as production of CO_2 (in grams per litre per hour) during first 3 days of fermentation. Produced volatile acidity was expressed as concentration of acetic acid. Volatile acids were measured by the OIV reference method, using steam distillation and acid-base titration [15].

Re-identification of yeast isolates

Molecular identification

DNA was isolated from 2 ml of an overnight-grown yeast culture (YD medium, aerobic conditions, orbital shaker at 2 Hz, 28 °C, initial cell concentration approximately 10^5 $CFU \cdot ml^{-1}$) by chaotropic solid-phase extraction using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The internal transcribed spacers (ITS) of 5.8S rDNA gene regions were amplified by polymerase chain reaction (PCR) using primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) [16]. DNA amplification was carried out in the final volume of 50 μl, the reaction mixture containing 1× reaction buffer for the DNA polymerase, 1 μl of template DNA, 1.5 U HotStarTaqPlus DNA polymerase (Biotium, Hayward, California, USA), 4 $mmol \cdot l^{-1}$ $MgCl_2$, 0.8 $mmol \cdot l^{-1}$ of each dNTP and 500 $nmol \cdot l^{-1}$ of each primer. PCR programme was as follows:

initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and polymerization at 72 °C for 1.5 min, final polymerization at 72 °C for 10 min. PCR products were detected by electrophoresis in 10 $g \cdot l^{-1}$ agarose (Merck) gel. The gels were stained by GelRed (Biotium) and visualized under UV light. A 250 bp DNA ladder size marker (Invitrogen, Carlsbad, California, USA) served as the size standard. The PCR products were purified and sequenced for both strands by a commercial facility (Microsynth, Balgach, Switzerland). The obtained sequences were compared with those present in the GenBank database using a BLAST search (National Center for Biotechnology Information, Bethesda, Maryland, USA). Sequences for the 5.8S-ITS rDNA region with ≥99% nucleotide identity were considered to correspond to the same species.

Identification by MALDI-TOF MS

Yeast isolates after ethanol-formic acid extraction were identified by MALDI-TOF MS as previously described [17] with some modifications. The strains were grown on YDA at 28 °C overnight. Each strain was analysed in triplicate. MALDI-TOF MS analysis was performed on an Autoflex mass spectrometer (Bruker Daltonik, Bremen, Germany) with the MALDI Biotyper version 3.1 software (Bruker Daltonik) and analysed by the standard pattern-matching algorithm, which compared the spectrum acquired to those present in the library. The results of the pattern-matching process were expressed as log score values, which ranged from 0 to 3. Score values between 2.300 and 3.000 were considered as highly probable species identification; score values between 2.000 and 2.299 were considered as genus identification and probable species identification; score values between 1.700 and 1.999 were considered as probable genus identification and score values below 1.7 were considered as no reliable identification.

RESULTS AND DISCUSSION

Isolation and taxonomic identification

In the present work, *Saccharomyces cerevisiae* yeasts were isolated from 12 sites in the Czech Republic and Slovakia. In total, 1634 axenic yeast cultures were isolated from 20 samples of grapes. From this, 231 strains (14%) were selected on the basis of morphology of cell and sporulation (formation of *Saccharomyces*-type asci). These strains were purified using a micromanipulator with pneumatic microinjector. Afterwards, cell morphology and vegetative reproduction of isolated strains was examined. The shape of yeast cells was globose to ovoid with diameters of (3–10) × (3–10) μm. The globose shape of cell has many advantages for the purpose of alcoholic fermentation. Such shaped strains are more tolerant to osmotic pressure and, therefore, are more suitable for production of wines with a higher concentration of ethanol [18]. Budding of yeast cells was multilateral. All strains formed unconjugated persistent asci. It was formerly reported [19] that ascospore formation of *S. cerevisiae* was usually below 10%, except in highly fertile homothallic strains where sporulation ranged from 40–95%. Sporulation of the studied strains ranged from 40 % to 55 %.

Afterwards, taxonomic tests were carried out. The tested key characteristics of species assigned to the genus *Saccharomyces* are presented in Tab. 3. None of the strains was able to grow at 40 °C as had been shown for some pathogenic *S. cerevisiae* strains [20]. Only 3% of all isolated strains were identified as *S. cerevisiae* (Tab. 4).

Screening of oenological properties

In order to determine oenological properties of the isolated strains, several tests were carried out with the 48 strains identified as *S. cerevisiae* (Tab. 5). General technological parameters for selection of wine yeasts are defined by OIV [11]. From the wide spectrum of characteristics, only some, which we had considered important for application, were chosen [21].

Resistance to high osmotic pressure during fermentation is a key requirement in winemaking. All tested strains were able to ferment in the presence of 35% (w/w) glucose, while almost half of the strains were tolerant to 50% (w/w) glucose. Six strains were able to ferment in the presence of 55% (w/w) glucose. More than two thirds of strains grew in the presence of 17.5% (v/v) of ethanol and almost half of them even in the presence of 20% (v/v) of ethanol.

Potential of production of hydrogen sulphide (H_2S) was tested by inoculating the yeasts on BiGGY agar. It was found previously that only 61% of wild S*accharomyces* strains produce H_2S [22]. All isolated strains in our research produced H_2S at least on a minimal level (1 – cream colonies). Only two strains were considered low producers, namely, RRB5 and PGI67. The colony colour of most isolates was from light brown (2) to brown (3).

Modern criteria of yeast selection include analysis of enzyme activities. For detection of β-glucosidase activity, semi-quantitative test using

Tab. 3. Key fermentation and growth characteristics of strains.

Strain ID	Fermentation								Growth						
	Glc	Gal	Sac	Mal	Lac	Raf	Tre	Mel	In	Mel	Gly	Man	VF	37 °C	40 °C
RRB5	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
WRD70	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PAE58	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PAE59	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PAG64	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PAG65	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
DUH135	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
DUH136	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PAH47	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PAH48	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
DEH53	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
DEH54	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PGI66	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PGI67	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
PNI68	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
MTJ61	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
MTJ62	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
RRK152	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
RRK153	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
GTL106	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
GTL107	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
GTL108	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
GTL109	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
GTL110	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
GTL111	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
GTL112	+	+	+	+	–	+	–	–	–	–	–	–	–	+	–
RRA3	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
SBC28	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
RRC29	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
RRC30	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
RRC31	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
RRC32	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
RRC33	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
PAC34	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
PAC35	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
PAC36	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
RRE60	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
BFF14	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
BFF15	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
BFF16	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
BFF17	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
PAG63	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
DUH133	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
DUH134	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
WRH25	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
WRH26	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
MMH45	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–
MMH46	+	–	+	+	–	+	–	–	–	–	–	–	–	+	–

All data are average of three replicates.
Glc – glucose, Gal – galactose, Sac – saccharose, Mal – maltose, Lac – lactose, Raf – raffinose, Tre – trehalose, Mel – melibiose, In – inulin, Mel – melibiose, Gly – glycerol, Man – mannitol, VF – vitamin-free medium, 37 °C – growth at 37 °C, 40 °C – growth at 40 °C, (+) – positive, (–) – negative.

Tab. 4. Axenic yeast cultures isolated.

Sample ID	Number of axenic yeast cultures					
	All yeasts	*Saccharomyces*-like strains	All *S. cerevisiae*	Strain ID	Selected *S. cerevisiae*	Strain ID
RRA	86	17	1	RRA3	1	RRA3
RRB	90	12	1	RRB5	1	RRB5
SBC	84	10	1	SBC28	1	SBC28
RRC	64	12	5	RRC29, RRC30, RRC31, RRC32, RRC33	1	RRC33
PAC	76	10	3	PAC34, PAC35, PAC36	1	PAC34
WRD	79	8	1	WRD70	1	WRD70
PAE	81	11	2	PAE58, PAE59	1	PAE58
RRE	86	11	1	RRE60	1	RRE60
BFF	97	14	4	BFF14, BFF15, BFF16, BFF17	1	BFF14
PAG	95	14	3	PAG63, PAG64, PAG65	1	PAG63
DUH	112	19	4	DUH133, DUH134, DUH135, DUH136	1	DUH135
WRH	87	10	2	WRH25, WRH26	1	WRH25
MMH	66	8	2	MMH45, MMH46	1	MMH45
PAH	56	9	2	PAH47, PAH48	1	PAH48
DEH	69	9	2	DEH53, DEH54	1	DEH53
PGI	78	8	2	PGI66, PGI67	1	PGI67
PNI	68	9	1	PNI68	1	PNI68
MTJ	65	6	2	MTJ61, MTJ62	1	MTJ62
RRK	97	18	2	RRK152, RRK153	1	RRK152
GTL	98	16	7	GTL106, GTL107, GTL108, GTL109, GTL110, GTL111, GTL112	1	GTL112
Total	1634	231	48		20	

Several strains were isolated from each sample. First selection was carried out based on morphological and physiological characteristics. Second selection was carried out based on oenological properties.

media containing esculin was used. It was formerly shown that results obtained by screening with this medium correlated to the glycerol-glucose indexes measured after fermentation, hence to the potential activity in real wine production conditions [23]. In our research, all tested strains displayed β-glucosidase activity. These results are in contrast with some previous studies [23, 24]. For production of white wines from aromatic vine varieties, it is preferred to use the strains with higher β-glucosidase activity. In contrast, for the red wine production it is preferable to choose a strain with lower β-glucosidase activity. There is a general lack of information on the effects of β-glucosidase activity of *S. cerevisiae* strains on grape and wine anthocyanins. Some studies showed that some wild strains of *S. cerevisiae* with β-glucosidase activity can decrease the anthocyanins in final wine. When selecting the yeast strain as a starter for production of red wine, β-glucosidase activity has to be carefully taken into account to avoid colour quality loss [25, 26].

During the growth in liquid medium in stationary conditions, various yeasts may form different structures. *S. cerevisiae* usually produces only a sediment and weak turbidity. Good sedimentation properties of cultures allow quick purification of young wines and predestine these yeasts even for use by secondary fermentation. The appearance of the culture in the liquid medium was evaluated after 3 days of incubation in YD medium at laboratory temperature. All strains sedimented well and formed bounded sediment without surface structures.

Fermentation rate is one of the most important properties used for the selection of wine yeasts. All strains displayed fermentation rate of a satisfactory level, i.e. ≥ 0.25 g·l^{-1}·h^{-1} of produced CO_2. The rates were similar to results of previous studies, which were 0.59–1.56 g·l^{-1}·h^{-1} [25] and ≥ 1.25 g·l^{-1}·h^{-1} [26].

Acetic acid is a fermentation by-product that causes wine defects when present at high concentrations. Volatile acids detected after fermentation in microfermentation trials varied from 0.45 g·l^{-1} to 0.87 g·l^{-1} (expressed as acetic acid). Total concentration of volatile acids in wine usually varies from 0.2 g·l^{-1} to 0.8 g·l^{-1}. Slovak standards allow

Tab. 5. Technological characteristics of isolated yeast strains.

Strain ID	RRA3	RRB5	SBC28	RRC29	RRC30	RRC31	RRC32	RRC33	PAC34	PAC35	PAC36	WRD70	PAE58	PAE59	RRE60	BFF14	BFF15	BFF16	BFF17	PAG63	PAG64	PAG65	DUH133	DUH134
Selection for further application	+	+	+	–	–	–	–	+	+	–	–	+	+	–	+	+	–	–	–	+	–	–	–	–
Etanoltolerance [%] (v/v)	15.0	17.5	12.5	15.0	15.0	17.5	17.5	17.5	17.5	17.5	17.5	17.5	20.0	20.0	20.0	20.0	17.5	20.0	17.5	15.0	15.0	20.0	17.5	17.5
Osmotolerance [%] (w/w)	40	35	40	50	45	40	45	45	40	40	40	40	50	45	45	55	50	50	50	45	45	50	50	50
Production of H_2S	2	1	2	2	2	2	2	2	2	3	3	2	2	3	2	2	3	2	3	2	3	2	2	3
β-Glucosidase activity	1	3	2	3	2	1	2	3	3	2	2	2	3	2	2	1	2	2	1	3	2	2	3	3
Growth in liquid medium	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Fermentation rate [$g \cdot l^{-1} \cdot h^{-1}$]	1.22	1.02	1.24	1.12	0.94	0.86	1.12	1.37	1.44	1.16	1.14	1.32	1.27	0.73	1.54	1.42	1.11	1.42	1.54	1.32	0.92	1.12	1.02	1.08
Volatile acids production [$g \cdot l^{-1}$]	0.52	0.45	0.55	0.74	0.62	0.82	0.68	0.48	0.52	0.65	0.62	0.66	0.55	0.45	0.62	0.45	0.65	0.48	0.50	0.55	0.87	0.50	0.64	0.74

Strain ID	DUH135	DUH136	WRH25	WRH26	MMH45	MMH46	PAH47	PAH48	DEH53	DEH54	PGI66	PGI67	PNI68	MTJ61	MTJ62	RRK152	RRK153	GTL106	GTL107	GTL108	GTL109	GTL110	GTL111	GTL112
Selection for further application	+	–	+	–	+	–	–	+	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	+
Etanoltolerance [%] (v/v)	20.0	17.5	20.0	17.5	20.0	20.0	20.0	20.0	15.0	15.0	20.0	12.5	17.5	12.5	17.5	17.5	15.0	17.5	20.0	20.0	20.0	20.0	17.5	20.0
Osmotolerance [%] (w/w)	50	50	55	50	50	50	50	50	40	40	55	40	55	35	45	45	40	40	50	45	45	55	50	55
Production of H_2S	2	3	2	2	2	3	3	2	2	3	2	1	2	3	2	2	2	2	3	3	2	3	2	2
β-Glucosidase activity	1	3	2	1	3	3	2	3	3	3	2	2	1	2	3	2	2	3	3	3	3	3	3	3
Growth in liquid medium	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Fermentation rate [$g \cdot l^{-1} \cdot h^{-1}$]	1.32	1.28	1.33	1.42	1.33	1.25	1.32	1.43	1.42	1.23	0.91	1.20	0.87	0.93	1.38	1.39	1.23	0.86	1.23	1.17	1.04	1.31	1.22	1.46
Volatile acids production [$g \cdot l^{-1}$]	0.62	0.85	0.52	0.45	0.68	0.85	0.62	0.51	0.60	0.75	0.62	0.62	0.51	0.85	0.61	0.49	0.62	0.75	0.62	0.86	0.64	0.55	0.69	0.50

Production of H_2S (potential of production on Biggy agar): 0 – white colonies, no hydrogen sulphide production; 1 – cream colonies; 2 – light brown colonies; 3 – brown colonies; 4 – dark brown or black colonies, very intensive hydrogen sulphide production.
β-Glucosidase activity (semiquantitative test on media containing esculin): 0 – no activity; 1 – weak activity, 0–5 mm; 2 – moderate activity, 5–10 mm; 3 – strong activity, ≥ 10 mm.
Growth in liquid medium (flocculation or production of sediment): (+) – positive, (–) – negative.
Volatile acids production (expressed as acetic acid) and fermentation rate were determined after microfermentation on synthetic yeast extract-dextrose medium, relative standard deviation did not exceed 10 % of individual values.

the concentration of 1.1 $g \cdot l^{-1}$ for white still wines and 1.2 $g \cdot l^{-1}$ for red still wines.

Selection of yeast strains was carried out in a way that one strain from each sample was selected. Selection parameters were high osmotolerance (at least tolerance to 30 % w/w of glucose and 12.5 % v/v of ethanol) and fermentation strength, optimal growth in liquid media (no turbidity, no surface structures, good sedimentation), low production of H_2S and low production of volatile acids. β-Glucosidase activity was also evaluated depending on whether the yeast was intended to be used to produce white or red wine. For red wines, the higher β-glucosidase activity was taken as inappropriate.

Re-identification of yeast isolates

Traditionally, the identification and classification of yeasts was based on morphological, physiological and biochemical traits. This methodology requires evaluation of 60–90 tests, which need experienced microbiologists and are time-consuming [27]. As an improvement, various faster modern techniques were developed for yeast identification [28]. To confirm the conventional identification, selected strains were submitted to two modern identification methods (Tab. 6). It was previously demonstrated that analysis of 5.8S-ITS rDNA is a reliable technique for routine differentiation of yeasts at species level [9, 29, 30]. The ITS1-5.8S rDNA-ITS2 region of 20 strains was obtained by amplification with ITS1 and ITS4 primers. The PCR products of all the tested strains, when analysed by gel electrophoresis, showed a single band of 840–880 bp (Fig. 2), characteristic of strains belonging to the *Saccharomyces* genus. The PCR products were sequenced for both strands and the sequences were analysed by BLAST. Sequencing

Tab. 6. Results of identification by conventional tests, sequencing of ITS1-5.8S rDNA-ITS2 region and MALDI-TOF MS.

Strain ID	Conventional tests	ITS-sequence (percentage of homology)	MALDI-TOF MS (score)
RRA3	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.208) #
RRB5	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.143) #
SBC28	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.184) #
RRC33	*Saccharomyces cerevisiae*	No significant similarity found	*Saccharomyces cerevisiae* (2.172) #
PAC34	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.286) #
WRD70	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (1.844)
PAE58	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (1.955)
RRE60	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.173) #
BFF14	*Saccharomyces cerevisiae*	No significant similarity found	*Saccharomyces cerevisiae* (2.228) #
PAG63	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.402) *
DUH135	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.377) *
WRH25	*Saccharomyces cerevisiae*	No significant similarity found	*Saccharomyces cerevisiae* (2.283) #
MMH45	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (1.920)
PAH48	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.497) *
DEH53	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.360) *
PGI67	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (99 %)	*Saccharomyces cerevisiae* (2.045) #
PNI68	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.097) #
MTJ62	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.309) *
RRK152	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (99 %)	*Saccharomyces cerevisiae* (2.236) #
GTL112	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* (100 %)	*Saccharomyces cerevisiae* (2.235) #

ITS-sequence – internal transcribed spacer region sequence. Sequences for the 5.8S-ITS rDNA region with 99% nucleotide identity or higher were considered to correspond to the same species.
MALDI-TOF MS – matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Score values between 2.300 and 3.000 indicated highly probable species identification (*); score values between 2.000 and 2.299 indicated highly probable genus identification and probable species identification (#); score values between 1.700 and 1.999 indicated probable genus identification; score values below 1.7 indicated no reliable identification.

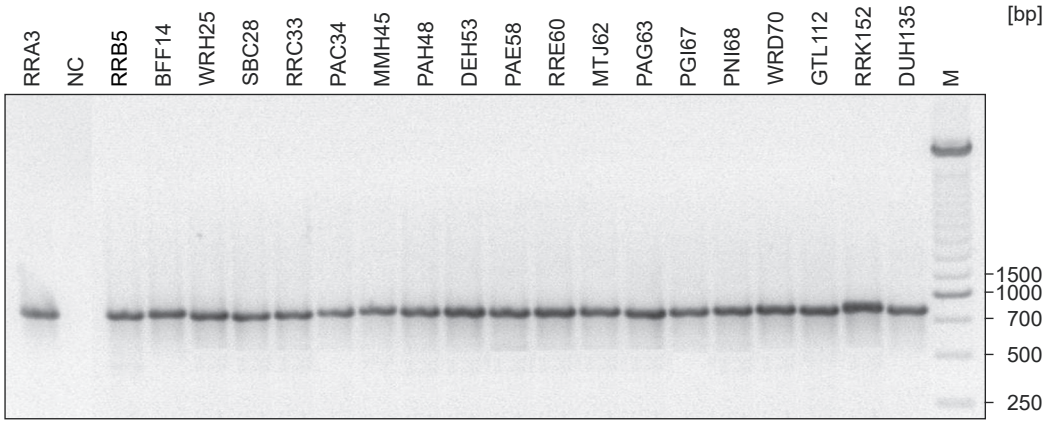

Fig. 2. PCR products of the ITS1-5.8S rDNA-ITS2 region.
Lanes: M – molecular size marker $n \times 250$ bp, NC – negative control.

of the ITS1-5.8S rDNA-ITS2 region of yeasts confirmed the results of conventional tests for 17 out of 20 strains.

Another identification test was performed by MALDI-TOF MS analysis, MALDI Biotyper database being used to identify the 20 yeast strains. The log score values of tested strains for these matches are listed in Tab. 6. The database of the manufacturer was used and the maximum log score values were obtained by five *S. cerevisiae* strains that were successfully identified on species level. Twelve strains presented scores between 2.000 and 2.299, which is the manufacturer-recommended cutoff for genus-level identification. The rest had a score between 1.700 and 1.999, which indicates probable genus identification. These results confirmed the data of several studies, where almost 50 % of analysed strains of *S. cerevisiae* reported log score values confirming only the genus [31]. In other studies [32, 33], an optimized sample preparation protocol was devised to increase the success of identification. Some strains in our study were not identified due to the absence of a reference spectrum in the database. After matching the correct taxon for these strains using molecular biology approaches, the spectra concerning the missing species need to be added in a supplementary database.

Five isolated strains were reliably identified as *S. cerevisiae* by 3 independent methods. Identity of strains RRC33, BFF14 and WRH25 was not verified by sequencing of the ITS1-5.8S rDNA-ITS2 region and it will be necessary to complete their identification by a different method. Using MALDI-TOF MS, only 5 strains were reliably verified as *S. cerevisiae.* Compared to sequencing of ITS1-5.8S rDNA-ITS2 region, MALDI-TOF MS is a very easy, quick and low-cost method. However, only with an extensive in-house library can MALDI-TOF MS become a powerful tool for rapid, easy and accurate identification of new yeast strains.

CONCLUSIONS

In this work, we compared the power of conventional and novel methods for identification of autochthonous yeasts isolated from vineyards in Czech Republic and Slovakia. For isolation of axenic cultures, a micromanipulator with a pneumatic microinjector was used. Axenic cultures were identified by conventional taxonomic key and this identification was verified by ITS sequencing and by MALDI-TOF MS. To select the most suitable strains as a starter culture for winemaking, several approaches were needed. All the selected yeasts showed a moderate potential of H_2S production, satisfactory mode of growth in liquid medium and a satisfactory fermenting power. From all isolated strains, those with suitable β-glucosidase activity, high ethanoltolerance, high osmotolerance and low volatile acids production were selected. Finally, selection of 20 yeast strains for possible use as a starter culture in the production of typical wines was carried out. Seven of them were isolated from newly-bred varieties originating in the Czech Republic and Slovakia. This paper demonstrates a useful approach for a fast selection of wine yeasts possessing interesting technological traits. Selection of specific autochthonous yeasts is important for winemakers wishing to take full

advantage of the potential of their regional territory for improving the wine production or making novel products.

Acknowledgements

This work was supported by Slovak Research and Development Agency (APVV-15-0333), by Grant Agency of Ministry of Education of Slovak Republic (VEGA 1/0063/18) and by Building Infrastructure for Modern Research of Civilization's Diseases (ITMS 26230120006) obtained from the Slovak Research Agency. The authors are grateful to Prof. Fedor Malík for his assistance in strain selection and critical reading of the paper.

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Received 28 June 2018; 1st revised 13 August 2018; accepted 21 August 2018; published online 4 December 2018.