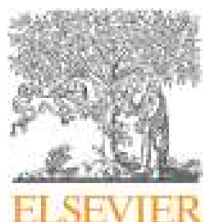

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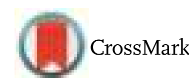
Food Microbiology

Isolation, identification and characterization of regional indigenous *Saccharomyces cerevisiae* strains



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ABSTRACT

In the present work we isolated and identified various indigenous *Saccharomyces cerevisiae* strains and screened them for the selected oenological properties. These *S. cerevisiae* strains were isolated from berries and spontaneously fermented musts. The grape berries (Sauvignon blanc and Pinot noir) were grown under the integrated and organic mode of farming in the South Moravia (Czech Republic) wine region. Modern genotyping techniques such as PCR-fingerprinting and interdelta PCR typing were employed to differentiate among indigenous *S. cerevisiae* strains. This combination of the methods provides a rapid and relatively simple approach for identification of yeast of *S. cerevisiae* at strain level. In total, 120 isolates were identified and grouped by molecular approaches and 45 of the representative strains were tested for selected important oenological properties including ethanol, sulfur dioxide and osmotic stress tolerance, intensity of flocculation and desirable enzymatic activities. Their ability to produce and utilize acetic/malic acid was examined as well; in addition, H_2S production as an undesirable property was screened. The oenological characteristics of indigenous isolates were compared to a commercially available *S. cerevisiae* BS6 strain, which is commonly used as the starter culture. Finally, some indigenous strains coming from organically treated grape berries were chosen for their promising oenological properties and these strains will be used as the starter culture, because application of a selected indigenous *S. cerevisiae* strain can enhance the regional character of the wines.

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Introduction

The quality of fermented foods and beverages is partially determined by the microorganisms used for their production. For instance, the secondary character of wine is determined by sensory characteristics that arise from the direct action of microorganisms on the substrate. The fermentation of grape must into wine is an ecologically complex process, in which bacteria and other microorganisms, especially yeasts, play a

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crucial role. The strains of *Saccharomyces cerevisiae* involved in fermentation play an important part in the characteristics of the final product and the diversity of *S. cerevisiae* strains present in spontaneous fermentation contribute to the chemical composition and sensory qualities of the resulting wine.[1]

Therefore, one of the most important technological advances in wine-making was the inoculation of grape juice with selected cultures of *S. cerevisiae*.[2] This approach is based on the evidence that microbiological control of the fermentation process allows better management of this alcoholic fermentation. It is known that selected strains of *S. cerevisiae* suppress indigenous non-*Saccharomyces* species and dominate the fermentation process.[3–5]

Nowadays, novel biotechnological approaches in winemaking are used in several aspects of the fermentation industry. Apart from the monitoring of the microbial populations and the control of the spoilage yeasts, attention is focused also on the selection and utilization of the starter cultures coming from one's own vineyard, which can enhance the regional character of the wine.[6,7] The metabolic peculiarities and the physiological properties of *S. cerevisiae* yeast may lead to the formation of metabolites and the transformation of grape substances that may enrich the wine flavor.[7] Certain criteria need to be met in order to guarantee the desirable features of the yeast strains selected. The most important ones are: tolerance to ethanol; growth at high sugar concentrations; resistance to sulfur dioxide; low production of hydrogen sulfide; production of killer toxins or some enzymatic activities.[8]

Furthermore, only reliable and rapid identification of the yeast species during the process and the quality control enables enologists to assess the role of yeasts as a main protagonist of alcoholic fermentation or as a contaminant. The utilization of molecular methods enabled rapid and precise identification of the yeasts at the species or strain level.[1] Mercado et al.[9] reported that *Saccharomyces* populations are represented by multiple strains, even in inoculated fermentations. Therefore, it is important to have simple and appropriate methods that allow discrimination at the strain level.

This study is focused on indigenous *S. cerevisiae* strain (i) selection, (ii) identification and (iii) technological characterization. Yeasts were isolated from grapes and musts during the production of wines. We selected two types of wine varieties – Sauvignon blanc and Pinot noir coming from an organic and integrated treated vineyard situated in South Moravia, Czech Republic. Our objective was also focused on the selection of the identification approaches that will be simple and suitable for rapid and reliable strain identification. Therefore, isolates of *S. cerevisiae* were identified and grouped by several molecular approaches such as *ITS*-PCR-RFLP, PCR-fingerprinting, species-specific primers and interdelta PCR typing. The combination of these techniques enabled rapid detection, identification and typing of different *S. cerevisiae* strains. Finally, the isolated strains were screened for selected technological properties important in the winemaking process and for further application as the starter cultures. To sum up, this study has demonstrated the importance of selection of an appropriate and rapid identification technique and also determination of some important oenological properties.

Methods

Yeast species isolation and cultivation

Autochthonous (indigenous) strains belonging to *Saccharomyces* genus were isolated from grape berries and also from spontaneously fermented musts in different stages of the fermentation process.

Grapes were collected at the Ivaň vineyard situated in the South Moravia region and belong to the Mikulov wine region, Czech Republic. Red wine Pinot Noir (Pn) and white wine Sauvignon blanc (Sg) cultivars of *Vitis vinifera* were chosen. Both varieties, typical cultivars for white and, respectively, red Moravian wine production, were cultivated using the organic (O) and also integrated (I) farming procedure. Grape berries (healthy and undamaged) were collected before harvest (in September 2009, 2010, 2011) into sterile glasses. Immediately after transportation to the laboratory, 15–20 grape berries were placed into 150 mL of Malt extract medium MEM (Himedia; 2% malt extract, 0.1% peptone, 2% dextrose, 2% agar) and cultivated for 10 days at laboratory temperature (approx. 23 °C). After that, culture media (300 μL) inoculated onto MEM agar plates supplemented with 250 mg L^{-1} streptomycin sulfate (Himedia, India) were incubated at 26 °C for 3–5 days. The single colonies were obtained by Koch's dilution method.

The fermentation process was performed in the cellar in Ivaň (during the vintage 2009, 2010, 2011), which was separated from common fermentations of commercial wine production. The fermentation process following the standard procedure was conducted in a 1000 L barrel. The cellar temperature was approx. 10 °C and the temperature of the fermentation was approx. 18 °C. The must was spontaneously fermented and the samples were collected 3 times per week during the whole fermentation process.

Yeast populations from must were isolated as described previously[10] and cultivated on malt extract medium (MEM) supplemented with 250 mg L^{-1} streptomycin sulfate (Himedia, India). The single colonies (pure culture) were obtained by Koch's dilution method.

In total, 120 *Saccharomyces* sp. strains were isolated and identified. Pure cultures were preserved on MEM agar under paraffin oil. The most promising strains will be deposited in the yeast culture collection CCY Bratislava (strain *S. cerevisiae* 1-09 has already been deposited there).

DNA isolation

Genomic DNA was isolated from single colonies using the commercial kit UltraClean™ Microbial DNA Isolation Kit (MoBio, USA) according to the manufacturer's protocol.

ITS-PCR-RFLP

To distinguish *Saccharomyces* sp. from other isolates, ITS-PCR-RFLP was employed. The internal transcribed spacers (*ITS*) (ITS1 and ITS2) and 5.8S rDNA gene regions were amplified by using specific primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[11] DNA amplification was carried out in the final volume of 50 μL containing

0.2 mM of dNTP, 0.5 μL of each primer 100 pmol μL^{-1}, 1× PCR reaction buffer and 1 U of *Taq* DNA polymerase (Kapa Biosystems, USA). PCR conditions were as follows: initial denaturation cycle at 94 °C for 4 min followed by 25 cycles of amplification, denaturation at 94 °C for 1 min, annealing at 48 °C for 30 s, and extension at 72 °C for 1 min; final extension at 72 °C for 10 min.

For RFLP, PCR products were purified by ethanol precipitation and digested by restriction endonucleases *Hae*III (Fermentas, USA) following the manufacturer's instructions.

Species-specific primers

For *S. cerevisiae* species identification, a set of pairs of species-specific primers ScerF2 (5′-GCGCTTTACATTCAGATCCCGAG-3′) and ScerR2 (5′-TAAGTTGGTTGTCAGCAAGATTG-3′) were used.[12] DNA amplification was carried out in a final volume of 25 μL containing 0.2 mM of dNTP (Invitek, Germany), 0.5 μL of each primer 100 pmol μL^{-1} (GeneriBiotech, Czech Republic), 1× PCR reaction buffer, 1.5 mM $MgCl_2$ and 1.25 U *Taq* DNA polymerase (BioRad, USA) and 0.5 μL of template DNA. PCR cycling conditions used were: initial denaturation cycle at 94 °C for 4 min followed by 30 cycles of amplification, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; final extension at 72 °C for 2 min.

PCR-fingerprinting and interdelta PCR typing

In order to distinguish different *S. cerevisiae* strains, PCR-fingerprinting by using M13 primer and interdelta primers were used. DNA amplification was carried out in a final volume of 25 μL containing 0.2 mM of dNTP (Invitek, Germany), 0.5 μL of each primer 100 pmol μL^{-1} (VBC Biotech, Germany), 1× PCR reaction buffer and 1 U *Taq* DNA polymerase (Kapa, USA) and 0.5 μL of template DNA.

PCR-assays by using M13 primer (5′-GAGGGTGGCGGTTCT-3′):[13] initial denaturation cycle at 95 °C for 5 min, followed by 40 cycles of amplification followed by denaturation at 93 °C for 0.75 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min; final extension at 72 °C for 6 min. The second PCR-assay for M13 primer: initial denaturation cycle at 94 °C for 4 min, followed by 35 cycles of amplification followed by denaturation at 94 °C for 30 s, annealing at 36 °C for 45 s, and extension at 72 °C for 45 s; final extension at 72 °C for 7 min.

The conditions for interdelta PCR typing by δ1 (5′-CAAAATTCACCTATATTCTCA-3′) and δ2 (5′-GTGGATTTTTATTCCAACA-3′); δ2 (5′-GTGGATTTTTATTCCAACA-3′) and δ12 (5′-TCAACAATGGAATCCCAAC-3′)[14,15] were as follows: initial denaturation cycle at 94 °C for 4 min, followed by 30 cycles of amplification–denaturation at 94 °C for 30 s, annealing at 49 °C for 1 min and extension at 72 °C for 2 min. The final extension was at 72 °C for 10 min.

Detection of PCR products

PCR products and restriction fragments were separated and detected by electrophoresis on 2% (w/v) agarose gel in 1× TBE buffer at 5 V cm^{-1} for 2 h. DNA amplified by single repetitive primers and by delta primers were separated on 1.5% agarose gel for 3–4 h. The gels were stained by ethidium bromide (10 mg L^{-1}), visualized under UV light (Ultra Lum. Inc., USA) and documented by ScionImage software (Scion, India). Finally, electrophoregrams were processed by the BioNumerics 6.5 software employing UPGMA cluster analysis.

Screening of oenological properties

Flocculation properties of selected strains were tested according to Bony et al.[16] with slight modification. Yeasts were cultured for 3 days at 26 °C in tubes containing 10 mL of YPD (2% peptone, 1% yeast extract and 2% glucose) medium under permanent shaking (150 rpm). Cells were collected by centrifugation and washed with deionized water. After that, cells were suspended into 10 mL of 50 mM acetate buffer (pH 4.5) enriched by 3 mM $CaSO_4$. The tubes of suspended cells were mixed for 30 s and the turbidity of yeast suspension was evaluated by the naked eye. Flocculation degree was determined using a subjective scale which means that the sedimentation was observed depending on the time. The following evaluation was used: + the cells flocculate and sediment after 15 min (partially clear solution); ++ immediate flocculation; w – flocculation after 1 h.

Ethanol tolerance was tested in 5 mL of YPD medium supplemented by 12, 14, 15, 16 and 17% (v/v) ethanol and the tubes were inoculated by 100 μL of cell suspension (cell concentration approx. 5 log CFU mL^{-1}). Inoculated tubes were cultivated at 26 °C for 10 days. The culture density was measured daily by DEN-1B densitometer (Biosan, Latvia). Specific growth rate μ (h^{-1}) and the length of the lag phase t (h) were estimated.

Osmotolerance of selected strains was tested in 5 mL of YPD medium with 40% (w/v) and 50% (w/v) glucose. The cells density was measured as described above.

H_2S production by selected strains was tested on Biggy agar (Himedia, India) which contains bismuth as an indicator. After incubation at 26 °C for 3–5 days, the zone surrounding the colony was evaluated as the follows: − no production; + white colonies; ++ light brown; +++ brown; ++++ dark brown/black.[5]

Malic and acetic acid utilization by selected strains was tested on 0.67% yeast nitrogen base (YNB, Himedia) agar plates containing 0.5% (w/v) malic acid, respectively, 0.25% (w/v) acetic acid. The growth of colonies was screened. Acetic acid production was screened on $CaCO_3$ agar plates (Custer's chalk medium) containing 0.5% yeast extract, 5% glucose, 0.5% $CaCO_3$ and 2% agar. The acetic acid production enabled solubility of $CaCO_3$ which resulted as a clear zone surrounding the colonies.[5]

The isolated yeasts were also tested for some enzymatic activities such as β-glucosidase and glycosidase activities. The activities were determined by agar plating. The plates were incubated at 26 °C for 3–5 days.[17]

β-Glucosidase activity was screened onto selective medium containing 0.67% yeast nitrogen base (YNB, Himedia), 0.5% arbutin and 2% agar. The pH of the medium was adjusted to 5 before autoclaving. Two milliliters of a filter sterilized 1% ferric ammonium citrate solution was added to 100 mL media before plates pouring. Colonies showing activity were identified by a dark brown halo around the colonies.[17]

Table 1 – Number of *Saccharomyces* sp. isolates.

Grape variety	Vintage	Number of *Saccharomyces* sp. isolates	
		Integrated (I) Grapes (B)/must (M)	Organic (O) Grapes (B)/must (M)
Sauvignon blanc (Sg)	2009	1/14	1/11
Pinot noir (Pn)	2010	0/14	0/17
Sauvignon blanc (Sg)	2011	0/20	0/17
Pinot noir (Pn)	2011	1/14	0/10
Total		120	
Total of yeasts isolates		524	

Glycosidase activity was determined using the plates with the selective medium containing 0.67% yeast nitrogen base (YNB, Himedia), 0.2% rutin and 2% agar. The glycosidase activity was detected as a clear zone around the colonies.[5,17]

Results and discussion

Selection and identification of S. cerevisiae strains

In total, we isolated 120 single colonies of autochthonous *Saccharomyces* sp. strains from 524 total yeasts. Yeasts were isolated from Sauvignon blanc (Sg) and Pinot noir (Pn) coming from organic (O) and integrated (I) farming. The yeast species were isolated from grape berries as well as from spontaneously fermented must during the vintage 2009 (09), 2010 (10) and 2011 (11). The list of the *Saccharomyces* isolates and the sources of their isolation are shown in Table 1.

Yeasts of the genus *Saccharomyces* were distinguished from the other isolates (from non-*Saccharomyces* species) by *ITS*-PCR-RFLP (the length of PCR amplicon was 880 bp). Further, for *S. cerevisiae* species identification we employed species-specific primers ScerF2 and ScerR2.[12] Species-specific primers enable us to identify and distinguish *S. cerevisiae* species from other species belonging to the *Saccharomyces sensu stricto* complex, which includes the species which can also be found in fermented must (for. ex. *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces kudriavzevii*). Based on our results, all the isolates belonging to the *Saccharomyces* genus were identified by species-specific primers as *S. cerevisiae* (the length of the PCR products was 150 bp) (data not shown).

Further, two different PCR-assays by using M13 primer were used. These assays differed in annealing temperature (50 °C vs. 36 °C). The isolates were divided into three groups by the first assay with annealing temperature 50 °C and into four groups by the second PCR-assay (36 °C). Data are presented as part of the dendrogram (Fig. 1). Two isolates (marked as U, T) exhibited a different fingerprinting profile than the rest of the isolates; these isolates may be hybrids of *S. cerevisiae* and another species belonging to the *Saccharomyces* genus. Hence, PCR-fingerprinting techniques using M13 primer are able to group the species members of *Saccharomyces* genus but they are not suitable for resolving.

In order to distinguish various *S. cerevisiae* strains, we employed δ1–δ2 and δ12–δ2 primers amplifying inter-delta sequences. These delta elements are described as appropriate genetic markers for identification of polymorphisms because the number and location have a certain intraspecific variability.[18] The primer pair δ1–δ2 generated from three to eight different fragments per strain with one common band of the size of approximately 1000 bp. On the other hand, primer pair δ12–δ2 provided significantly more fragments per sample and some of them were of low intensity. The low number of fragments per sample is ascribed to the weak homology exhibited by the primer δ1–δ2 towards the whole sequence of *S. cerevisiae* genom.[19] However, by combination of these two sets of delta primers, we identified 45 different *S. cerevisiae* strains. The majority of the isolated and identified *S. cerevisiae* strains came from spontaneous fermented musts and belonged to group A (strain A). Despite the fact that some authors[20,21] reported that it is almost impossible to isolate *Saccharomyces* sp. populations from berries and initial must by standard direct agar plating procedure due to their low counts (>10–100 CFU cm^{-2}) we isolated three strains from grape berries (see Table 1). The electrophoretic patterns and final dendrogram showing the genetics similarity of various identified *S. cerevisiae* strains are shown in Fig. 1.

The novel combination of the modern molecular approaches used in this study for strain identification and typing seems to be suitable for rapid, reliable, simple and reproducible identification of *S. cerevisiae* strains. Schuller et al.,[22] Maqueda et al.[23] or Ortiz et al.[24] reported application of mitochondrial DNA restriction analysis or karyotyping in order to distinguish various strains of *S. cerevisiae*. However, these methods are labor intensive and the results are influenced by complexity of data interpretation due to the high number of generated fragments after mtDNA RFLP. On the contrary, we employed different approaches to the group and distinguish *Saccharomyces* at strain level based on consequent employment of PCR-fingerprinting. For instance Schuller et al.[22] reported that interdelta PCR typing had very similar resolving power at strain level as mtDNA and karyotyping, therefore, the unique combination of the methods utilized in this work can be considered as a simple and rapid alternative to mtDNA or karyotyping.

Screening of selected oenological properties of various S. cerevisiae strains

Because not all yeast strains are relevant for the specific conditions and characteristics of wine, a number of criteria have been proposed for the selection of new yeast strains for use in the winemaking process. One of the most important criterions for high-quality wine production is use of *S. cerevisiae* strain with suitable technological properties. Thus, in order

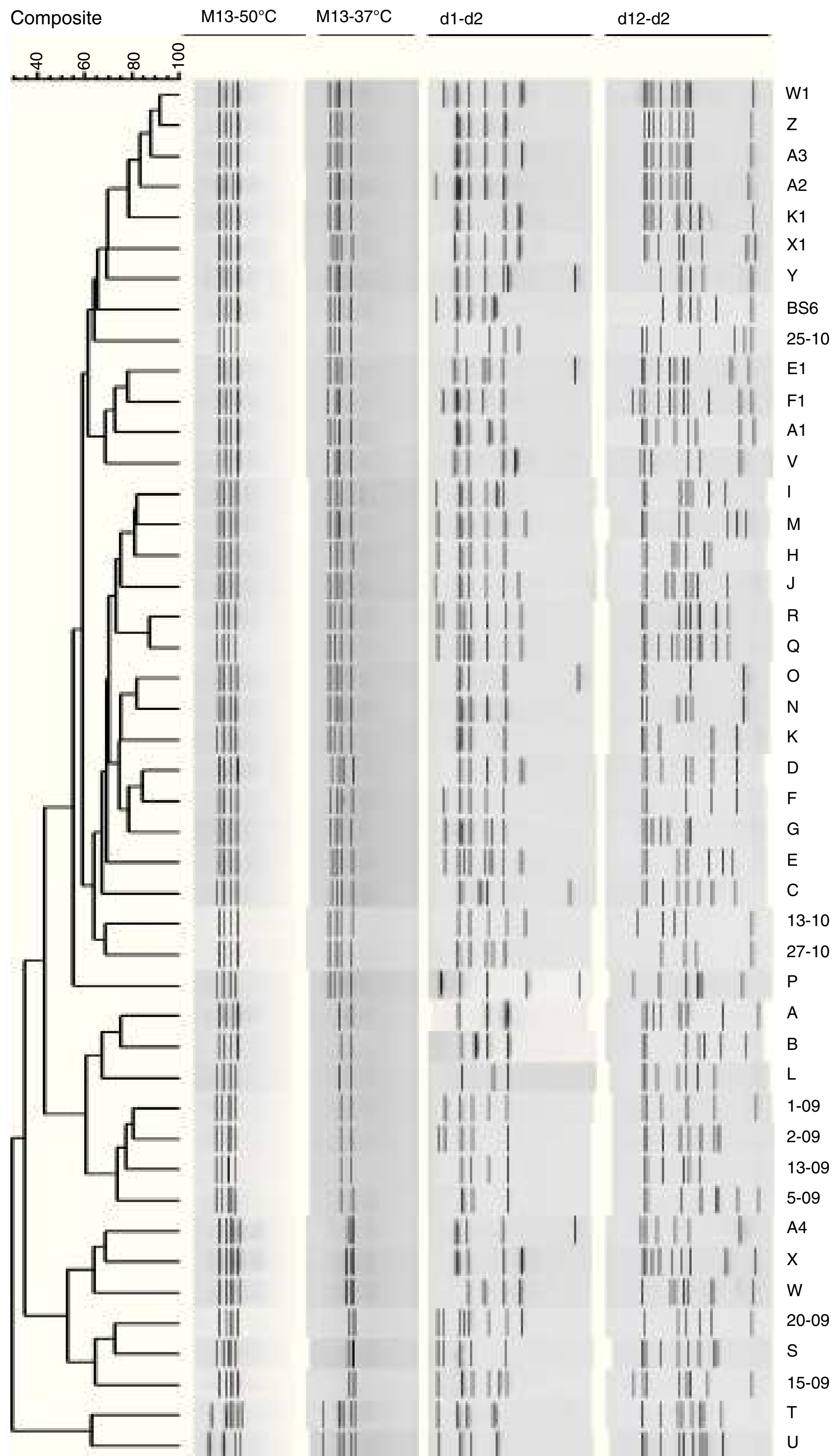

Fig. 1 – Dendrogram based on the similarity of PCR-fingerprinting patterns of *S. cerevisiae* isolates. *S. cerevisiae* BS6 – control strain. The description on the right side of the dendrogram is the label of the isolated *S. cerevisiae* strains.

Table 2 – Oenological properties of different indigenous *Saccharomyces cerevisiae* strains and one commercial *S. cerevisiae* strain BS6 as a control. Pn – Pinot Noir; O, I – organic, integrated; M, B – must, grape berries; 09–11 – mean year of isolation (2009–2011). The gray labelled fields – the strains which physiological properties were better than control strain BS6.

Isolated strain	Source of isolation	Desirable technological properties										
		Ethanol resistance (%)							Osmotolerance			
		12% ethanol		14% ethanol		Ethanol (%)			40% glucose		50% glucose	
		μ (h^{-1})[b]	lag phase t (h)	μ (h^{-1})[b]	lag phase t (h)	15	16	17	μ (h^{-1})[b]	lag phase t (h)	μ (h^{-1})[b]	lag phase t (h)
A	PnIB-11	0.061	22	0.019	58	+	–	–	0.037	19	0.029	24
A1	SgOM-11	0.054	22	–	–	–	–	–	0.044	19	0.029	24
A2	PnOM-11	0.058	24	0.025	59	+	–	–	0.049	19	0.021	24
A3	PnIM-11	0.055	24	–	–	–	–	–	0.037	28	0.013	24
A4	SgIM-11	0.092	24	0.017	39	–	–	–	0.052	19	0.022	24
B	PnOM-10	0.060	24	0.028	85	–	–	–	0.055	19	0.023	30
C	SgOM-09	0.060	24	0.033	58	–	–	–	0.057	19	0.020	24
D	PnOM-10	0.075	24	0.018	42	–	–	–	0.054	19	0.014	24
E	PnIM-10	0.080	22	0.013	42	–	–	–	0.051	19	0.020	43
E1	SgOM-11	0.057	24	0.033	80	–	–	–	0.051	19	0.020	24
F	SgOM-09	0.097	24	0.019	42	–	–	–	0.059	19	0.021	35
F1	SgIM-11	0.073	16	0.017	43	–	–	–	0.051	19	0.020	24
G	PnOM-10	0.058	24	0.018	45	–	–	–	0.065	19	0.021	28
H	SgIM-09	0.056	22	0.014	42	+	–	–	0.064	19	0.025	67
I	SgOM-09	0.090	22	0.063	24	–	–	–	0.052	19	0.019	51
J	PnOM-10	0.055	24	0.046	24	–	–	–	0.058	19	0.024	52
K	PnIM-10	0.061	14	0.034	68	–	–	–	0.060	19	0.016	43
K1	PnIM-11	0.065	24	0.032	68	–	–	–	0.060	24	0.022	45
L	SgIB-09	0.071	22	0.018	40	–	–	–	0.071	19	0.019	43
M	PnOM-10	0.061	14	0.017	40	+	w	–	0.051	19	0.023	51
N	PnIM-10	0.068	20	0.023	58	–	–	–	0.055	19	0.022	45
O	PnIM-10	0.055	20	0.005	150	–	–	–	0.061	19	0.028	67
P	PnOM-10	0.080	24	0.028	50	–	–	–	0.068	24	0.020	51
Q	SgOM-09	0.078	24	0.014	42	–	–	–	0.061	19	0.012	43
R	SgIM-09	0.060	24	0.013	24	–	–	–	0.062	19	0.018	43
S	SgIM-09	0.063	24	0.011	25	+	–	–	0.064	19	0.017	43
T	SgIM-09	0.070	24	0.010	24	+	w	–	0.065	19	0.017	43
U	SgIM-09	0.062	22	0.013	42	–	–	–	0.057	24	0.020	35
V	SgOM-11	0.049	4	0.010	38	–	–	–	0.064	19	0.022	51
W	SgIM-11	0.060	20	0.013	24	+	+	–	0.062	19	0.020	43

Table 2 – (Continued)

Isolated strain	Source of isolation	Desirable technological properties										
		Ethanol resistance (%)							Osmotolerance			
		12% ethanol		14% ethanol		Ethanol (%)			40% glucose		50% glucose	
		μ (h^{-1})[b]	lag phase t (h)	μ (h^{-1})[b]	lag phase t (h)	15	16	17	μ (h^{-1})[b]	lag phase t (h)	μ (h^{-1})[b]	lag phase t (h)
W1	PnIM-11	0.076	14	0.016	38	–	–	–	0.077	19	0.020	43
X	SgIM-11	0.064	24	0.018	42	–	–	–	0.072	19	0.022	43
X1	PnOM-11	0.075	25	0.016	25	+	+	–	0.064	19	0.018	51
Y	SgOM-11	0.073	22	0.006	24	–	–	–	0.065	19	0.022	67
Z	PnOM-11	0.064	16	0.007	24	–	–	–	0.058	19	0.018	43
1-09	SgOB-09	0.067	22	0.009	24	+	+	–	0.071	19	0.021	52
2-09	SgIM-09	0.058	24	0.011	40	+	+	–	0.064	19	0.016	55
5-09	SgIM-09	0.062	22	0.010	42	–	–	–	0.065	19	0.024	46
13-09	SgOM-09	0.058	16	0.008	24	–	–	–	0.063	19	0.021	51
15-09	SgOM-09	0.067	22	0.013	24	–	–	–	0.069	24	0.020	46
20-09	SgOM-09	0.059	22	0.006	26	+	w	–	0.062	19	0.019	46
13-10	PnOM-10	0.062	20	0.009	40	–	–	–	0.060	19	0.020	60
27-10	PnIM-10	0.065	24	0.003	80	–	–	–	0.065	19	0.021	44
25-10	PnIM-10	0.063	20	0.014	42	–	–	–	0.060	19	0.020	26
BS6	Control	0.064	20	0.012	24	–	–	–	0.070	24	0.018	26
Positive (%)		100	–	96	–	24	9	–	–	–	100	–
Weak (%)		–	–	4	–	–	7	–	–	–	–	–
Negative (%)		–	–	–	–	76	84	100	–	–	–	–

Isolated strain	Source of isolation	Desirable technological properties						Undesirable properties
		Osmotolerance						H_2S production
		SO_2 tolerance	MA utilization 0.5%	AA utilization 0.25%	AA production	Glycosidase activity	Flocculation	
A	PnIB-11	+	+	+	w	–	+	++
A1	SgOM-11	+	+	+	w	–	+	++
A2	PnOM-11	+	+	+	+	–	+	+++
A3	PnIM-11	+	+	+	+	–	+	++
A4	SgIM-11	+	+	+	+	–	+	++
B	PnOM-10	+	w	w	w	–	++++	–
C	SgOM-09	+	w	+	+	–	+	+++
D	PnOM-10	+	+	+	w	–	+	+++
E	PnIM-10	+	+	+	w	–	+	++++
E1	SgOM-11	+	+	+	+	–	+	++++
F	SgOM-09	+	+	+	+	–	+	+
F1	SgIM-11	+	+	+	w	–	+	++

Table 2 – (*Continued*)

Isolated strain	Source of isolation	Desirable technological properties						Undesirable properties
		Osmotolerance						H_2S production
		SO_2 tolerance	MA utilization 0.5%	AA utilization 0.25%	AA production	Glycosidase activity	Flocculation	
G	PnOM-10	+	+	+	w	–	+	++++
H	SgIM-09	+	+	+	+	–	w	++++
I	SgOM-09	+	+	+	++	–	+	++
J	PnOM-10	+	+	+	++	–	+	++
K	PnIM-10	+	w	+	++	–	+	++
K1	PnIM-11	+	w	+	w	–	+	++
L	SgIB-09	+	+	+	w	–	+	++
M	PnOM-10	+	+	+	++	–	+	+
N	PnIM-10	+	+	+	w	–	+	++++
O	PnIM-10	+	+	+	w	–	+	++++
P	PnOM-10	+	+	+	w	–	+	+
Q	SgOM-09	+	+	+	–	–	+	+
R	SgIM-09	+	+	+	w	–	+	+
S	SgIM-09	+	+	+	+	–	+	+
T	SgIM-09	+	+	+	–	–	+	++
U	SgIM-09	+	+	+	–	–	+	++
V	SgOM-11	+	+	+	–	–	+	++
W	SgIM-11	+	+	+	–	–	+	+
W1	PnIM-11	+	+	+	–	–	+	++++
X	SgIM-11	+	+	+	–	–	+	+
X1	PnOM-11	+	+	+	w	–	+	+
Y	SgOM-11	+	+	+	w	–	+	+
Z	PnOM-11	+	+	+	+	–	+	+
1-09	SgOB-09	+	+	+	w	–	+	+
2-09	SgIM-09	+	+	+	w	–	+	+
5-09	SgIM-09	+	+	+	–	–	+	++++
13-09	SgOM-09	+	+	+	–	–	w	++
15-09	SgOM-09	+	+	+	–	–	+	++++
20-09	SgOM-09	+	+	+	–	–	+	+
13-10	PnOM-10	+	+	+	–	–	w	+++
27-10	PnIM-10	+	+	+	–	–	+	++
25-10	PnIM-10	+	+	+	–	–	+	++
BS6	Control	+	+	+	–	–	+	++
Positive (%)		100	91	98	29	–	91/2	9[a]/20
Weak (%)		–	9	2	38	–	7	31/38
Negative (%)		–	–	0	33	100	–	2

Note: no glucosidase and glycosidase activities for all tested indigenous strains and also for commercial BS6 strain.

[a] Strong positive; for H_2S production: positive (+++ and ++++), weak (+ and ++).

[b] Specific growth rate; the standard deviations were not higher than 20%.

to investigate phenotypic differences among 45 indigenous *S. cerevisiae* strains, which were identified by molecular method as different strains (see above), we screened them for the selected technological properties. The strain *S. cerevisiae* BS6, which is commercially available, was used as a control strain. The complete list of results, summarizing oenological properties that are important for strain selection and their application in the winemaking process, is provided in Table 2. The use of local, autochthonous, selected strains of *S. cerevisiae* as starters is rather preferable, since these yeasts are better acclimated to particular conditions characteristics for the specific region/area of wine production[8] and, moreover, utilization of the local isolate of *S. cerevisiae* is likely to raise the regional character of the wine.

Barrajón et al.[25] reported that high fermentation power is obviously related to the capacity of the strain to overcome the stress associated with wine fermentation. For this purpose, we tested osmotic and ethanol stress tolerance.

The major target of ethanol is the membrane, altering the membrane organization and permeability and consequently inhibiting glucose transport and fermentation rate under enological conditions.[26] As expected, the physiological differences among the stress tolerant species *S. cerevisiae* depended on the strain. The growth parameters of all the tested strains including specific growth rate and length of the lag phase are listed in Table 2. Eighteen isolated strains revealed better growth characteristics than control strain BS6 in the presence of 12% ethanol and 29 showed higher growth rate when exposed to 14% ethanol. Further, four isolated strains (W, X1, 2-09, 1-09) were able to grow in 16% ethanol but none of the tested strains was able to grow in the presence of 17% ethanol.

Higher sugar content of the grape must can result in inhibition of the yeast metabolism and, therefore, in sluggish fermentation.[24] Therefore, tolerance towards osmotic pressure is a desirable property of the yeast starter culture. All the isolated strains were capable of growing in the presence of 40% and 50% glucose. Four strains (W1, X, L and 1-09) exhibited higher growth rate than the control strain BS6 in the presence of 40% glucose. Interestingly, despite the fact that Tofalo et al.[26] did not notice any growth of *S. cerevisiae* strains in the presence of 50% glucose, most of our isolates were capable of growing when cultivated in the presence of 50% glucose. Moreover, the majority of the screened strains revealed a higher growth rate on 50% glucose than the control strain.

Results of flocculation tests showed that, as also reported in other studies,[24,27] most of the strains (91%) remained in suspension after 10 min at rest. This is an important feature when selecting active dried yeasts for vinification, where yeast should ideally remain in suspension during fermentation.[24]

Enzymes play a definitive role in the production of wine. The enzymatic activities do not only originate from the grapes itself, but also from yeasts and other microorganisms.[17] Based on our results, tested strains lacked β-glucosidase activity. Unlike the other authors,[5,28] we did not detect any glycosidase production by *S. cerevisiae* strains tested.

To produce a high-quality wine, it is important to obtain a fine balance between the various chemical constituents, especially between the sugar and acid content.[29] As reported by Suárez-Lepe and Morata,[7] yeasts might be selected for their ability to produce and degrade acetic and malic acid. Our results showed that 91% (98%) of our isolates were able to grow on the agar medium containing malic or acetic acid as sole carbon sources. The capability of degradation of malic as well as acetic acid may be considered as a desirable property of yeast strains because it leads to the deacidification of wine.[30] On the contrary, acetic acid production was observed for 29% of tested strains. Several studies have linked the production of acetic acid to increased glycerol production which gives the wine a desirable property.[31,32]

Hydrogen sulfite has negative organoleptic impact on wine due to formation of off-flavors.[7] Fifteen isolates (29%) synthesized H_2S and remaining strains exhibited only low H_2S production. Only one strain (B) did not produce H_2S, however, this strain is not suitable for application as a starter culture due to strong flocculation properties.

Yeast selection offers the best way to obtain strains of *S. cerevisiae* or other oenological species with properties that might improve the sensorial profile, technological properties or regional character of the wine.[7] The assays to determine yeast properties that could influence fermentative capacity and the ability to adapt to stressful conditions related to the wine-production process revealed differences for some strains. Some indigenous strains exhibited better adaption to stressful conditions than the control strain. On the contrary, some of them produced a high amount of hydrogen sulfite, causing off-flavor.

Thus, if we compare all the tested strains, only 15 (A, A4, F, F1, I, J, L, M, R, S, X1, Z, 1-09, 2-09, 27-10) of them are suitable for further testing as starter cultures. Based on our results, these strains showed proper technological properties that are very similar to the control commercial *S. cerevisiae* BS6 strain. These strains can be chosen for future large-scale fermentation processes instead of commercially available strains.

Conclusions

The present study demonstrated application of appropriate modern molecular techniques that are suitable for rapid *S. cerevisiae* strain identification and further testing of various strains for their technological potential. The combination of used modern molecular techniques including species-specific primers, and interdelta PCR typing enabled us to identify *S. cerevisiae* at strain level. Also important physiological characteristics of the yeasts used in this study are suitable for rapid selection of the different *S. cerevisiae* strains that can be applied in the winemaking process. Application of the selected strains with suitable technological properties in the wine fermentation process should increase the quality of the wine and enhance the regional character of traditional Moravian wines. Hence, some isolated indigenous strains will be tested in a large-scale fermentation process by a small Moravian winery.

Conflicts of interest

The authors declare no conflicts of interest.

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