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SPONTANEOUS FERMENTATION IN WINE PRODUCTION AS A CONTROLLABLE TECHNOLOGY

Radim Holešinský, Božena Průšová, Mojmír Baroň, Jaromír Fiala, Petra Kubizniakova, Vít Paulíček, Jiří Sochor

ABSTRACT

This study focuses on the isolation of a consortium of microorganisms from spontaneously fermenting must that naturally contain lactic acid bacteria, non-saccharomyces yeasts, and saccharomyces yeasts. To collect the greatest diversity of microorganisms, the consortium was taken from the point of micro-sparkling. Based on the growth curves, isolation was performed using individual special nutrient media, and the isolates were subsequently multiplied in the nutrient medium. Individual isolates were then used for fermentation tests to monitor the percentage of fermented sugar and hydrogen sulphide production. The highest fermentation abilities were achieved in the isolates containing *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. The smallest amount of ethanol was formed from the isolates containing *Hanseniaspora uvarum*, while *Candida sake* isolate produced the lowest amount of hydrogen sulphide and *Zygosaccharomyces bailii* produced the highest. The other isolates produced an average amount. Based on these results, a consortium containing the given isolates in a certain ratio was compiled.

Keywords: spontaneous fermentation; yeast cultivation; yeast isolation; growth curves

INTRODUCTION

Saccharomyces cerevisiae (*S. cerevisiae*) is a type of yeast that performs alcohol fermentation and is widely used as a fermentation starter. During the alcoholic fermentation of grape must, *S. cerevisiae* becomes the dominant species with the increasing ethanol concentration **(Mas, Guillamón and Beltran, 2016)**. Therefore, the isolation of natural *S. cerevisiae* is generally carried out from must be fermented by spontaneous fermentation **(Versavaud et al., 1995; Valero et al., 2007; Clavijo, Calderón and Paneque, 2010; Cordero-Bueso et al., 2011; Viel et al., 2017; Crosato et al., 2018)**, suggesting that *S. cerevisiae* is common on grapes. Recently, **Taylor et al. (2014)** reported that *Saccharomyces* sp. makes up less than 0.00005% of the yeast population on ripe grapes.

Similarly, **Fleet (2003)** reported its presence at concentrations below 10 – 100 cfu.g^{-1}, and **Fleet (2003)** and **Martini, Ciani and Scorzetti (1996)** reported that the total number of cells never exceeds 10 cfu.g^{-1} on grape berries (cfu - colony forming units).

The diversity and quantity on grapes always vary depending on the variety, stage of ripening, terroir, vintage, vineyard age, soil type, the geographical location of the vineyard, climatic conditions, diseases, pests, and vineyard work used **(Pretorius, 2000; Mannazzu, Clementi and Ciani, 2002; Valero et al., 2007; Barata, Malfeito-Ferreira and Loureiro, 2012; Setati et al., 2012; Bokulich et al., 2014)**.

Equally important is the secondary microbial process of malolactic fermentation (MLF), which is when malic acid is converted into nicer-tasting lactic acid. Lactic acid bacteria (LAB) are involved in this process **(Bauer and Dicks, 2004)**, and this microbial development is also associated with the release of other metabolites that are capable of affecting wine quality **(Campbell-Sills et al., 2016)**.

Model microorganisms for alcoholic and malolactic fermentation are *Saccharomyces cerevisiae* and *Oenococcus oeni*. The selected strains belonging to these two species have been used to design starter cultures that are useful for promoting alcoholic fermentation (AF) and MLF, thus making the fermentation process more manageable **(Garofalo et al., 2016; Berbegal et al., 2017; Petruzzi et al., 2017)**.

MLF can also occur spontaneously, but its course is often unpredictable. It can take place over several months after the end of AF or fail due to unfavorable conditions in a wine based on the wine's pH, ethanol, and SO_2 content **(Berbegal et al., 2016; Lucio et al., 2017)**.

Another risk of spontaneous MLF is the formation of undesirable by-products, colour reduction, and higher synthesis of biogenic amines **(Guo et al., 2015; Henríquez-Aedo et al., 2016)**.

Oenococcus oeni is generally the main species used as an MLF starter due to its easy adaptation to wine conditions. New strains such as *L. plantarum* are currently being discovered, however, that can also survive and adapt well to the viticulture. These strains also have more favorable biological properties compared to *O. oeni*, such as higher growth rate, creation of a more complex aromatic profile, and the prevention of undesirable by-product formation **(du Toit et al., 2011; Brizuela et al., 2017)**.

Therefore, the aim of this study was to isolate yeast and lactic bacteria from the vineyard and carry out a fermentation experiment using these isolates.

Scientific hypothesis

Each vineyard has its own wine of microbiota, which affects the quality of the resulting wine. Separation and cultivation methods can be used to characterization and multiplying individual microorganisms. Based on the fermentation and H_2S production tests, the resulting consortium can be assembled, which can be used for fermentation, thereby promoting both the fermentation process and the terroir of the wine.

MATERIAL AND METHODOLOGY

The aim was to obtain a functional consortium of wine microorganisms that was characterized in terms of biotechnology and taxonomy. This consortium was separately cultivated to achieve the required amounts and then used to inoculate a drained batch of Hibernal must in which fermentation and H_2S production were monitored.

Materials

MEA+T Malt Extract Agar with Tetracycline (a broad-spectrum antibiotic against both gram-positive G+ and gram-negative G- bacteria). Only eukaryotic microorganisms grow on this medium.

Composition: agar – 15 $g.L^{-1}$, malt extract – 30 $g.L^{-1}$, mycological peptone – 5 $g.L^{-1}$, (manufacturer: Sigma Aldrich)

WLN: Wallerstein Nutrient Agar for counting and culturing yeast and bacteria.

Composition: agar – 20 $g.L^{-1}$, bromocresol green – 0.022 $g.L^{-1}$, calcium chloride – 0.125 $g.L^{-1}$, casein enzymic hydrolysate – 5 $g.L^{-1}$, dextrose – 50 $g.L^{-1}$ (manufacturer: Sigma Aldrich).

MRS (Agar according to DeMan, Rogosa, and Sharpe): Nutrient agar for the determination of lactic bacteria.

Composition: Agar – 12 $g.L^{-1}$, diammonium bicarbonate – 2 $g.L^{-1}$, potassium hydrogen phosphate – 2 $g.L^{-1}$, D (+) – glucose – 20 $g.L^{-1}$, magnesium sulphate – 0.1 $g.L^{-1}$, manganese sulphate – 0.05 $g.L^{-1}$, meat extract – 5 $g.L^{-1}$, sodium acetate – 5 $g.L^{-1}$, universal peptone – 10 $g.L^{-1}$, yeast extract – 5 $g.L^{-1}$. (manufacturer: Sigma Aldrich)

YPD (Yeast extract Peptone Dextrose) agar: Solid medium for yeast multiplication.

Composition: Bacteriological peptone – 20 $g.L^{-1}$, yeast extract – 10 $g.L^{-1}$, glucose – 20 $g.L^{-1}$, agar – 15 $g.L^{-1}$. (manufacturer: Sigma Aldrich)

ME (M-enterococcus) agar: Agar consists of tryptose; yeast extract; glucose; disodium hydrogen phosphate; sodium azide; 2, 3, 5-triphenyltetrazolium chloride; agar; and distilled or deionized water. (manufacturer: Sigma Aldrich)

BIGGY (Bismuth Sulphite Glucose Glycine Yeast Agar): Selective and differential medium with addition of bismuth salt for H_2S detection. Bismuth reacts with the resulting sulfane to form a precipitate that colours the agar below the colony.

Composition: Glucose – 10.0 $g.L^{-1}$, glycine – 10.0 $g.L^{-1}$, bismuth ammonium citrate – 5.0 $g.L^{-1}$, sodium sulphite – 3.0 $g.L^{-1}$, yeast extract – 1.0 $g.L^{-1}$, agar – 13.0 $g.L^{-1}$. (manufacturer: Sigma Aldrich)

The must of the Hibernal variety: This variety was developed in Germany in 1944 as a hybrid of Seibel 7053 (Chancellor) and Riesling. The must be clarified by sedimentation (after 24 hours). The turbidity value of the must after clarifying was approx. 400 NTU and this was not adjusted. The sugar content was 16 °NM, pH was 3.51, titratable acid content was 6.48 $g.L^{-1}$, and assimilable nitrogen content was 321 $mg.L^{-1}$.

Isolation of yeasts

The must of the Hibernal variety was fermented spontaneously. During this spontaneous fermentation, 20 mL of the matrix (must, fermentation must, wine) was taken at selected monitoring points (must, micro-sparkling, 4 and 8 vol. % ethanol, end of fermentation). The sample obtained was subsequently diluted using the so-called decimal series. From each dilution, 250 mL was pipetted onto Petri dishes with MEA+T, WLN, and MRS culture medium, and a microbiological rod smear was performed. The Petri dishes were then placed in a thermostat (30 °C; WLN and MEA+T – 3 days; MRS – 7 days).

At the end of the cultivation, the total number of microorganisms and individual colonies was enumerated. Based on a combined analysis of phenotypic characters (macroscopic and microscopic properties), a sampling point was selected (the most suitable was the micro-sparkling point - the diversity of technologically important microorganisms was evaluated – saccharomyces and non-saccharomyces yeasts and lactic bacteria) to serve as a source for consortium acquisition.

Determination of growth characteristics of individual isolates of the 2018 Wine Microorganism Consortium (growth curves)

The aim was to determine the growth characteristics of individual isolates. Individual isolates of microorganisms were pre-cultured in standard media (yeast – YPD; lactic acid bacteria - MRS) with the following culture conditions: 30 °C; shaking 120 rpm; yeast 24 h; and lactic acid bacteria 72 h. The obtained cell suspensions were centrifuged (10 mins; 10 °C; 10,000 rpm), washed with saline solution, and then resuspended in the selected media (yeast – YPD, ME, YPD_{mod}; lactic acid bacteria – MRS, YPD, and YPD_{mod}) so that the resulting optical density value of the suspension was 0.2 at a wavelength of 600 nm.

The obtained suspension was then pipetted onto Bioscreen C culture plates (Oy Growth Curves Ab Ltd). Each arrangement (microorganism x medium) consisted of five repetitions to ensure the achievement of relevant results.

Table 1 Results of the operational microbiological monitoring of the fermentation process.

Sampling time interval	MEA+T (cfu.mL^{-1})	WLN (cfu.mL^{-1})	MRS (cfu.mL^{-1})
Must	3.80E +05	4.00E +05	4.00E +02
Micro-sparkling	1.75E +06	2.35E +06	7.00E +03
EtOH 4 Vol. %	8.00E +06	5.00E +06	5.00E +02
EtOH 8 Vol. %	9.00E +06	1.20E +07	1.20E +02
End of fermentation	5.00E +04	4.00E +04	0

Note: cfu - colony forming units.

The culture conditions of the Bioscreen C device were set as follows: 30 °C; shaking every 3 mins; duration of one shaking cycle = 1 min; and the so-called wide band of wavelengths – WB (420 – 620 nm). The maximum growth rate μ (h^{-1}) was then calculated from the measured data, and the maximum optical density OD_{MAX} was determined. These data, along with the course of growth curves, served to assign culture media to individual isolates.

Identification of microorganisms

The MALDI TOF (matrix-assisted laser desorption/ionization coupled with time of flight mass spectrometry) method was used to identify microorganisms. It is a very accurate, simple method, able to determine high molecular weight substances, proteins, peptides, lipids, nucleic acids, carbohydrates **(Huong et al., 2014)**.

An essential part of the MALDI TOF measurement was the preparation of fresh α-cyano-4-hydroxycinnamic acid. The organic solvent was prepared by mixing 500 μL of acetonitrile (100%), 475 μL of distilled water, and 25 μL of trifluoroacetic acid. Before use, 250 μL of organic solvent was added to the plastic tube. The contents of the tube were vortexed until the complete dissolution of the crystals. α-cyano-4-hydroxycinnamic acid was stored in the dark place and its preparation is ideal the day before the measurement.

The cultures were applied to the clean metal plate for MALDI TOF and the culture was allowed to dry on the plate. It was then covered with 1 microliter of α-cyano-4-hydroxycinnamic acid. At the same time, it was important to homogenize the sample and matrix **(Jarolímková, 2017)**.

Unlike the analysis of bacteria, preprocessing of the yeast isolates was required to extract fungal proteins. The protein extraction method used to process yeast isolates for MALDI-TOF MS was adapted directly from established methods used to identify difficult bacterial isolates. Specifically, 1 to 5 colonies of an isolate were inactivated in 75% ethanol, pelleted, and then suspended in a 1:1 mixture of 70% formic acid and acetonitrile. The resulting supernatant was then analyzed by MALDI-TOF MS **(Marklein et al., 2009; Bader et al., 2011; Dhiman et al., 2011)**. The results of the identifications are in Table 5.

Optaining pure cultures: Separation and lyophilisation

This procedure aimed to cultivate individual isolates of technologically important microorganisms and preserve them using the lyophilization method. Separate cultivation of individual microbial isolates was performed based on the information obtained from the growth characteristics. Individual taxa were first pre-cultured in 250 mL Erlenmeyer flasks (100 mL medium volume; orbital stirring 120 rpm; 20 °C). The media and the culture times are shown in (Table 3). The pre-cultured cell suspension was examined microscopically (cell morphology, elimination of contamination) and centrifuged (10,000 rpm; 10 mins; 10 °C).

After separating the supernatant, the pellet was washed with saline solution and re-centrifuged (10,000 rpm; 10 mins; 10 °C) and resuspended in the pure culture medium. The prepared suspension served as the inoculum for the second cultivation stage, which was carried out in 2,000 mL Erlenmeyer flasks (1,000 mL medium volume; orbital stirring 100 rpm; 30 °C). The media and the culture times used for the individual isolates are shown in (Table 5).

After the cultivation was complete, the suspension was repeatedly centrifuged and washed as described in the previous step. The obtained biomass was mixed with cryoprotective medium and shock-frozen (70 °C; 24 h). The frozen suspension was then lyophilized. The viability of the obtained dehydrated biomass was then determined, and according to the qualitative and quantitative microbiological analysis (Table 2) and the cell viability in the lyophilisate, the 2018 Wine Microorganism Consortium was compiled.

Fermentation tests using a consortium

Fermentation tests

Individual yeast isolates were initially cultured in 50 mL Erlenmeyer flasks (25 mL medium volume; orbital stirring 120 rpm; 30 °C). The media and the culture times used for the individual isolates are shown in (Table 5). In the obtained cell suspension, the cell density was determined by microscopic cell counting in a so-called Bürker chamber. The calculated amount of this suspension was then pipetted to a final concentration of 10^8 cells.mL^{-1} in a 250 mL Erlenmeyer flask (100 mL YPDm medium volume; without shaking; 25 °C). Fermentation was monitored by the gravimetric method, and weight loss due to the metabolic conversion of fermentable sugars to carbon dioxide and ethanol was observed.

H_2S production

Individual isolates were inoculated onto a BIGGY agar identification medium using a microbiological loop. Individual Petri dishes were statically cultured at 30 °C for 3 days. Based on the visual evaluation, the individual isolates were marked as low, medium, and high producers of H_2S.

BiGGY, Bismuth Sulphite Glucose Glycine Yeast Agar, is based on the formulation developed by **Nickerson (1953)** and mainly used for the isolation and presumptive identification of *Candida* species. In a study of sulphite

reduction by yeasts, the ability of many yeasts to reduce a bismuthyl hydroxy polysulphite was noted. Growth on an acidic or neutral medium containing bismuth sulphite produced black colonies because of the extra-cellular reduction of the bismuth sulphite, to bismuth sulphide. The bismuth sulphite complex confers a high degree of selectivity to the medium, and most strains of bacteria are inhibited on BIGGY Agar. In this study, BIGGY agar was used as a simple and rapid method to compare the rate of H_2S production between pure yeast isolates.

Statistical analysis

Statistical analyses and figures were generated using Excel 2007 software packages (manufactured by Microsoft Office, USA) and Statistica 10 statistical software (Copyright © StatSoft). The Statistica 10 software was used to process growth curves data and create their line graphs.

RESULTS AND DISCUSSION

Isolation of yeasts

The results of the microbiological analysis are shown in (Table 1). These data are comparable with the normal course of fermentation of the grape must. Based on the phenotypic analysis, a consortium was selected from the point of micro-sparkling.

The quantitative parameters of the individual taxa of the 2018 Wine Microorganism Consortium are given in (Table 2). The individual values in (Tables 1 and Table 2) were averaged from three measurements.

Through the application of microbiological techniques, the 2018 Wine Microorganism Consortium was obtained from the spontaneous batch, which was characterized in qualitative and quantitative terms.

Growth curves

The growth curve courses are shown in (Figures 1 and Figure 2), while the numerical parameters of the growth characteristics are shown in (Tables 3 and Tables 4). The growth characteristics of individual isolates were determined based on the growth curves of different types of media. These characteristics were used to assign culture media and culture times to individual isolates (Table 3).

Obtaining pure cultures: Separation and lyophilisation

To culture and lyophilise the 2018 Wine Microorganism Consortium, 30 g of the consortium was prepared and used for fermentation for 100 litres of must. The representation of the individual isolates can be seen in Table 4.

Table 2 Quantitative parameters of isolated taxa of the 2018 Wine Microorganism Consortium. Sampling from the point of micro-sparkling.

Isolate marking	Determined cell concentration (cfu.mL^{-1} of matrix)
LAB01	5.00E+03
LAB02	2.00E+03
Y01	3.50E+05
Y02	2.00E+05
Y03	1.20E+06
Y04	2.00E+05
Y05	1.00E+05
Y06	9.50E+05
Y07	1.10E+06

Note: cfu – colony forming units.

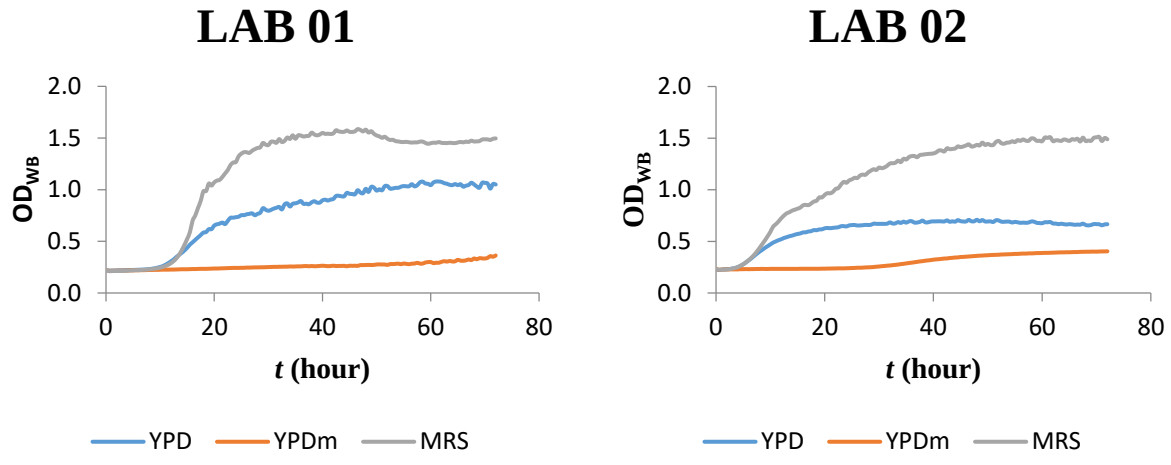

Figure 1 Growth curves of isolates LAB01 and LAB02; lactic acid bacteria.

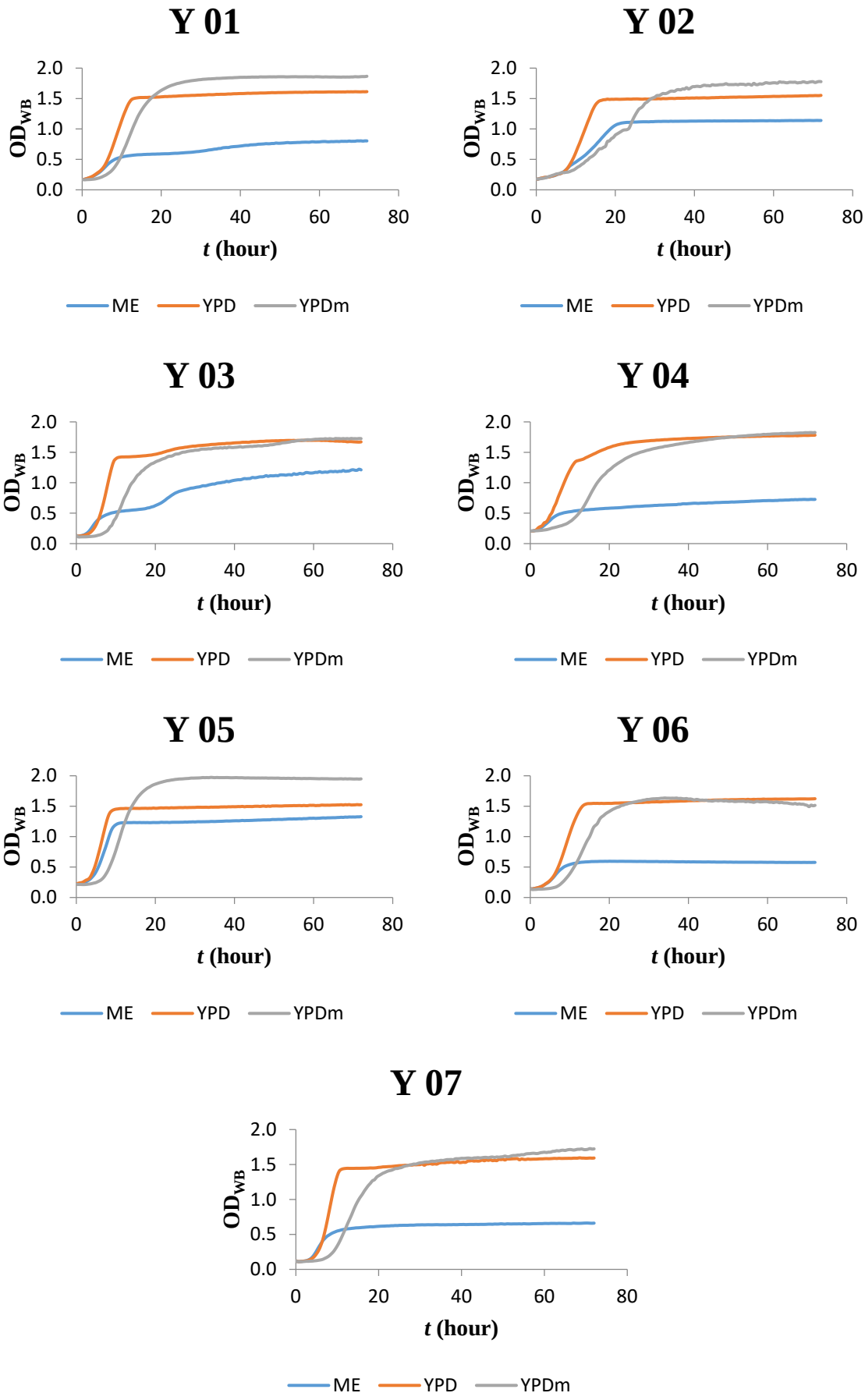

Figure 2 Growth curves of isolates Y01 – Y07; yeasts.

Table 3 Assignment of culture media and culture times to individual isolates.

Isolate marking	Culture medium	Cultivation time (h)
LAB01	MRS	32
LAB02	MRS	48
Y01	YPDm	22
Y02	YPDm	36
Y03	YPD	9
Y04	YPD	17
Y05	YPDm	24
Y06	YPD	12
Y07	YPD	10

Table 4 Weight composition of lyophilised 2018 Wine Microorganism Consortium preparation.

Isolates	Taxonomic identification
LAB01	*Lactobacillus brevis*
LAB02	*Lactobacillus plantarum*
Y01	*Hanseniaspora gulliermondi*
Y02	*Saccharomyces cerevisiae 1*
Y03	*Hanseniaspora uvarum 1*
Y04	*Hanseniaspora uvarum 2*
Y05	*Saccharomyces cerevisiae 2*
Y06	*Zygosaccharomyces bailii*
Y07	*Candida sake*

Table 5 Identification of individual isolates in the Consortium of Wine Microorganisms, 1 and 2 are different axenic cultures.

Isolates	Taxonomic identification
LAB01	*Lactobacillus brevis*
LAB02	*Lactobacillus plantarum*
Y01	*Hanseniaspora gulliermondi*
Y02	*Saccharomyces cerevisiae 1*
Y03	*Hanseniaspora uvarum 1*
Y04	*Hanseniaspora uvarum 2*
Y05	*Saccharomyces cerevisiae 2*
Y06	*Zygosaccharomyces bailii*
Y07	*Candida sake*

Table 6 Numerical parameters of fermentation tests of yeast isolates.

Isolate marking	Max. ethanol production rate (vol. %/day)	Max. EtOH concentration achieved (vol. %)
Y01	0.375	7.27
Y02	2.530	13.28
Y03	0.900	6.98
Y04	0.167	2.98
Y05	1.992	13.30
Y06	1.017	9.37
Y07	0.492	7.20

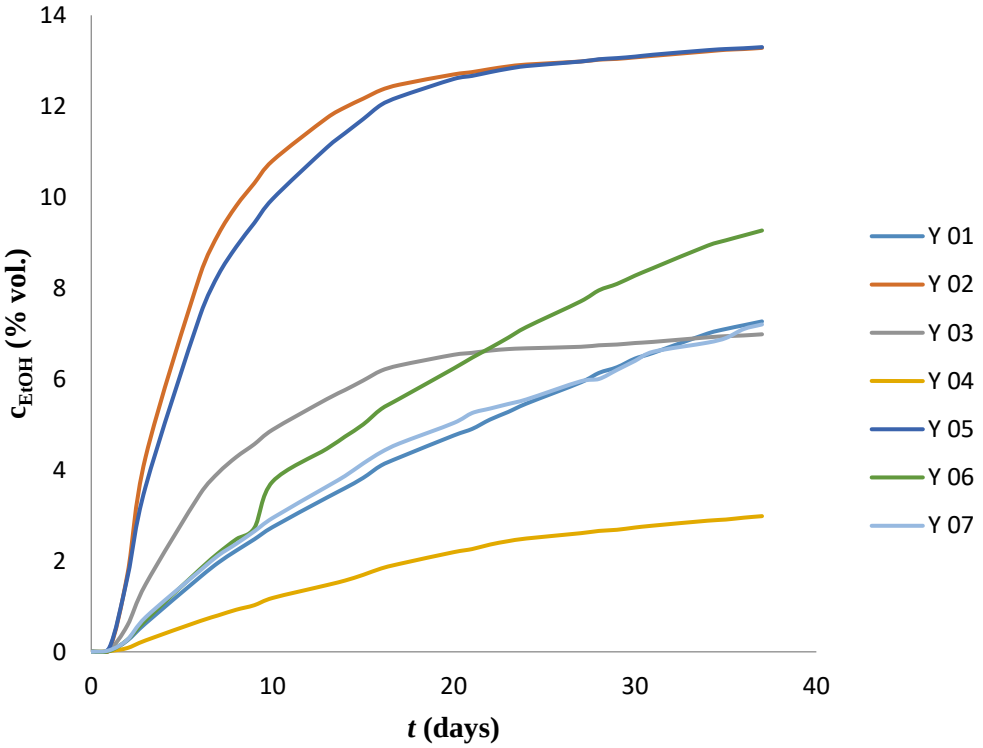

Figure 3 Course of fermentation tests of yeast microorganisms from the 2018 Wine Microorganism Consortium.

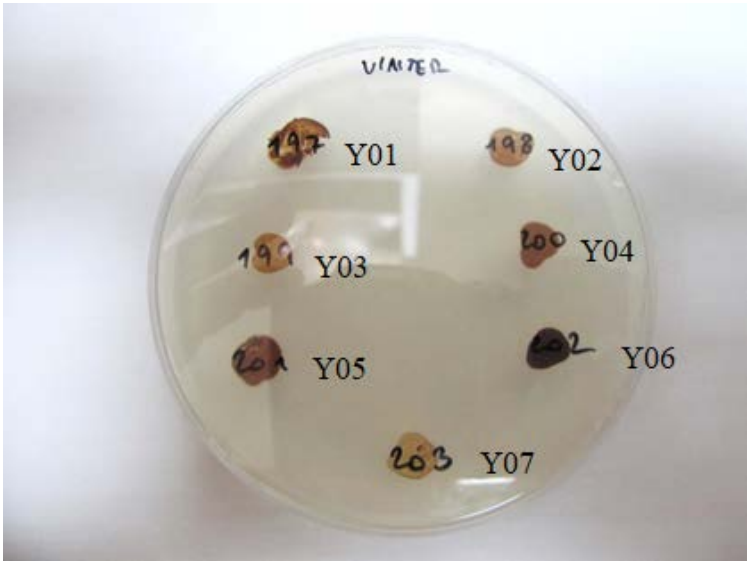

Figure 4 H_2S production by isolates Y01, Y02, Y03, Y04, Y05, Y06, and Y07.

Table 7 Evaluation of H_2S production by individual yeast isolates.

Isolate marking	H_2S production
Y01	Medium
Y02	Medium
Y03	Medium
Y04	Medium
Y05	Medium
Y06	High
Y07	Low

The growth curves, fermentation tests, and H_2S production results were used to inform the composition of the consortium so that the entire mixture exhibited the best fermentation capabilities, including the desired strains of lactic acid bacteria. Isolates Y03, Y06, and Y07 had the largest mass representation as characterized by a looser fermentation process but had the ability to ferment alcohol to 6.98, 9.37, and 7.20% vol. alc., respectively. These isolates showed low, medium, and high H_2S-producing ability. Isolates Y02 and Y05 had the second largest mass representation of the resulting consortium as characterized by high sugar fermentability up to an alcohol content of 13.28 and 13.30% vol. alc., respectively. The fermentation process was not gradual, however, which could negatively affect the quality of the wine. Both isolates showed mean H_2S production capacity. The isolate Y04 had a low mass representation. It showed the lowest sugar fermentability to an alcohol content of only 2.98 and had a medium ability to produce H_2S.

Table 5 contains the identification of individual isolates. The consortium consists of 2 strains of lactic acid bacteria and 7 strains of saccharomyces and non-saccharomyces yeast. The most abundant yeast strains of Consortium are *Hanseniaspora uvarum*, *Zygosaccharomyces bailii* and *Candida sake*.

Fermentation using isolates

Fermentation tests

Based on the results of the fermentation tests (Figure 3 and Table 6), we can conclude that there was some similarity between isolates from the 2018 Wine Microorganism Consortium. For example, between isolates Y02 and Y05 and isolates Y01 and Y07. For the Y03 and Y06 strains, there was always a certain deviation that distinguished them from the other isolates, and isolate Y04 showed a completely different course of fermentation compared to the other yeast microorganisms tested. Based on these data, we were also able to divide the strains into high fermentation strength strains (Y02 and Y05) and medium fermentation strength strains (Y01, Y03, Y06, and Y07).

Figure 3 shows the different fermentation progress of individual isolates. The consortium mixture was composed of isolates that showed different fermentation ability. The selection also included the incorporation of saccharomyces and non-saccharomyces yeast, which contribute to the sensory expression of the wine. Isolates Y03, Y06, and Y07 are characterized by a slower fermentation process. Isolates Y02 and Y05 were characterized by high sugar fermentability up to an alcohol content of 13.28 and 13.30% vol. alc., i.e. with a faster fermentation process. Y04 isolate showed the lowest sugar fermentability to an alcohol content of only 2.98, but the fermentation process was the most gradual.

H_2S production

Low, medium, and high sulfane productivity occurred during the monitoring of H_2S production by yeast isolates (Figure 4, Table 7). Most isolates (Y01, Y02, Y03, Y04, Y05) had medium sulfane productivity.

H_2S production was also monitored during the testing of a suitable fermentation strain, and strains with low, medium and high H_2S production were found. The Y07 strain showed low production; the Y01, Y02, Y03, Y04, and Y05 strains showed medium production; and the Y06 strain showed high production. Y02 and Y05 also showed high fermentation strength and medium H_2S production, while the Y07 strain produced a low amount of H_2S and had a medium fermentation capacity.

Discussion

This study focused on the isolation of yeasts and lactic acid bacteria representing the given vineyard. From the isolated microorganisms and growth curves, the most suitable culture media and required culture times were determined. The isolation was realized from the point of micro-sparkling of spontaneous fermentation due to the strain diversity. The study found that at an ethanol concentration stage of 4.5 – 5%, naturally, present non-saccharomyces yeasts die and ethanol-*tolerant S. cerevisiae* begins to act. Previous studies have also reported that many different strains occur at the beginning of fermentation, but only a few dominate in the later stages of wine fermentation **(Torija et al., 2001)**. Subsequently, fermentation tests were carried out to monitor the fermentation process and the fermentability of sugars to ethanol in individual isolates. Differences were found between individual isolates due to the isolation of microorganisms from the point of micro-sparkling when yeast diversity was greatest. During the fermentation tests, the differences in fermentability were shown, allowing us to select the yeast strain most suitable for fermentation.

Some similarities were found in the sugar fermentability of different isolates, specifically isolates Y02 and Y05 and Y01 and Y07. In the strains Y03 and Y06, there was always a certain deviation that distinguished them from the second group, and isolate Y04 showed a completely different course of fermentation compared to the other yeast microorganisms tested. Based on these data, we were also able to divide the strains according to strains with high fermentation strength (Y02 and Y05) and those with medium fermentation strength (Y01, Y03, Y06).

During spontaneous fermentation, different yeast species and strains interact with each other differently depending on the changing conditions of the fermenting must **(Albergaria and Arneborg, 2016; Ciani et al., 2016; Morrison-Whittle and Goddard, 2018)**. The medium becomes increasingly selective and this corresponds to the proportion of individual yeasts and bacteria **(Bisson, 2012; Perrone et al., 2013; Ciani et al., 2016; Brice et al., 2018; Henriques et al., 2018)**. Various studies indicate the prevalence of *S. cerevisiae* over non-saccharomyces, which usually initiate fermentation. *Saccharomyces* strains have greater tolerance to ethanol and temperature changes **(Goddard, 2008; Salvadó et al., 2011; Alonso-del-Real et al., 2017)**.

Ganucci et al. (2018) reported the effect of ethanol and temperature on the dominance of various *S. cerevisiae* strains occurring in multiple spontaneous fermentations carried out on an industrial scale. Another study by **Tofalo et al. (2013)** examined the prevalent strains of *S. cerevisiae*, which were differentiated by the RFLP-mtDNA method and according to their isolation frequency. The results obtained by an analysis of 637 isolates

confirmed the genetic polymorphism expected in the *S. cerevisiae* population in spontaneous wine fermentation and the high variability between isolation frequencies of different strains. **Schuller et al (2012)** evaluated intraspecific genetic diversity of fermentative vineyard-associated *S. cerevisiae* strains and evaluate relationships between grape varieties and geographical location on populational structures. Similar results are shown in the study **(Bisson, 2012; Schuller et al., 2012; Tofalo et al., 2013)**.

The study by **Ganucci et al. (2018)** further found that independent of the grape variety, five of the six wineries in the study only had one predominant *S. cerevisiae* strain with an isolation frequency ranging from 32 to 74%, while the variable number of strains (from four to 14) was characterized by an isolation frequency of less than 10%. This finding is consistent with those reported by other authors **(Versavaud et al., 1995; Gutiérrez et al., 1997; Egli et al., 1998; Sabate et al., 1998)**, although in some cases the predominant strains of *S. cerevisiae* were not found by the fermentation process **(Vezinhet et al., 1992)**.

To select the most suitable yeast strain for fermentation, H_2S production was also monitored on a special nutrient medium: BIGGY. The obtained isolates showed varying degrees of H_2S production, ranging from low, medium to high H_2S production. These results confirm those presented by **Perrone et al. (2013)** and **Pérez-Torrado et al. (2017)**, which state, inter alia, that the dominant behavior of yeast strains is due to differential H_2S production and killer factor resistance.

It is noteworthy that in the high-frequency strains that were tested by **Ganucci, Guerrini et al. (2018)**, no killer factor was detected and no significant differences in H_2S production were found. The degree of competition of each strain, which determines the ability of one strain to compete with another, is influenced by other factors, however, including pH, temperature, ethanol, osmotic pressure, and available nitrogen **(Ciani et al., 2016)**.

Ganucci et al. (2018) study looking at the effect of ethanol and temperature on growth performance and condition advantage of high-frequency *S. cerevisiae* strains showed that these two factors can play an important role in determining the dominance of one strain over another during wine fermentation. A single action of ethanol on the growth performance led to the high-frequency strains showing significantly lower inhibition than the low-frequency strains.

According to **Arroyo-López, Querol and Barrio (2009)**, an even more accurate indicator of total yeast growth is the percentage of inhibition as this parameter is indirectly related to the delayed phase but linearly related to both the maximum specific growth rate (μmax) and the maximum cell density at the end of growth. Consequently, there is an advantage of condition, which according to **Salvadó et al. (2011)**, represents the difference in μmax between competitors for specific environmental conditions. This leads to higher concentrations of high-frequency strains, indicating their enhanced adaptability to increasing ethanol concentrations compared to low-frequency strains. Each *S. cerevisiae* strain can exhibit different stress reactions to ethanol because the effects of increasing ethanol concentrations on the yeast cell include various changes, such as membrane composition and gene expression, synthesis of heat shock proteins, increase in chaperone proteins, etc.**(Ding et al., 2009)**.

Another study of four commercial wine yeast strains recently highlighted that fermentation temperature may be an important factor in determining the dynamics of a population of *S. cerevisiae* strains **(García-Ríos et al., 2014)**. Ethanol and high temperature synergistically affect membrane integrity and permeability, causing a decrease in yeast population growth **(Alexandre, Rousseaux and Charpentier, 1994; Albergaria and Arneborg, 2016)**.

CONCLUSION

The outcome of this study was the 2018 Wine Microorganism Consortium, which was obtained from the spontaneous fermentation that characterizes the given vineyard and supports the 'terroir' of the wine. At the same time, inoculation with this mixed culture helps to prevent problems with stagnant fermentation, which is often associated with spontaneous fermentation. Isolates of lactic acid bacteria and non-saccharomyces and saccharomyces yeasts were obtained and the resulting consortium was formed from these isolates. The obtained consortium was then used for fermentation tests where the percentage of fermented sugar and hydrogen sulphide production were monitored.

The Y03, Y06, and Y07 isolates had the largest mass representation and were characterized by a looser fermentation, but with the ability to ferment alcohol to 6.98, 9.37, and 7.20 vol. % alc. These isolates showed low, medium, and high ability to produce H2S. Y02 and Y05 isolates had the second largest mass representation of the resulting consortium and were characterized by high sugar fermentability up to an alcohol content of 13.28 and 13.30% vol. alc., but the fermentation process was not gradual, which could negatively affect the quality of the wine. Both isolates showed medium ability to produce H2S. Y04 isolate also has a low mass representation and showed the lowest ability to ferment sugar to an alcohol content of only 2.98 and a medium ability to produce H2S.

The Y03, Y06, and Y07 isolates had the largest percentage in the resulting consortium. These isolates formed 80% of the total weight of the consortium. The LAB 01 and LAB 02 isolates represented 7% of the consortium. Lactic acid isolates do not participate in alcoholic fermentation and are in the consortium to start malolactic fermentation.

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Contact address:

Radim Holešinský, Mendel University in Brno, Faculty of Horticulture, Department of Viticulture and Enology, Valtická 337, CZ-691 44 Lednice, Czech Republic, Tel. +420 721 412 888,
E-mail: holes.radim@gmail.com
ORCID: https://orcid.org/0000-0001-8966-3254

*Božena Průšová, Mendel University in Brno, Faculty of Horticulture, Department of Viticulture and Enology, Valtická 337, CZ-691 44 Lednice, Czech Republic, Tel.: +420 519 367 259,
E-mail: rusova.bozena@email.cz
ORCID: https://orcid.org/0000-0003-2582-1713

Mojmír Baroň, Mendel University in Brno, Faculty of Horticulture, Department of Viticulture and Enology, Valtická 337, CZ-691 44 Lednice, Czech Republic, Tel.: +420 519 367 252,
E-mail: MojmirBaron@seznam.cz
ORCID: https://orcid.org/0000-0003-1649-0537

Jaromír Fiala, Research Institute of Brewing and Malting, Lipova 15, Prague 12044, Czech Republic, Tel.: +420 224 900 126,
E-mail: fiala@beerresearch.cz
ORCID: https://orcid.org/0000-0002-7196-4523

Petra Kubizniakova, Research Institute of Brewing and Malting, Lipova 15, Prague 12044, Czech Republic, Tel.: +420 224 900 152,
E-mail: kubizniakova@beerresearch.cz
ORCID: https://orcid.org/0000-0003-2070-1616

Vít Paulíček, EPS biotechnology, s.r.o., V Pastouškách 205, 686 04 Kunovice, Czech Republic, Tel.: +420 777 743 542,
E-mail: vit.paulicek@epsbiotechnology.cz
ORCID: https://orcid.org/0000-0002-7632-7419

Jiří Sochor, Mendel University in Brno, Faculty of Horticulture, Department of Viticulture and Enology, Valtická 337, CZ-691 44 Lednice, Czech Republic, Tel: +420 519 367 254,
E-mail: jiri.sochor@mendelu.cz
ORCID: https://orcid.org/0000-0001-7823-1544

*Corresponding author