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Characterization of *Saccharomyces* Strains Isolated from "Kéknyelű" Grape Must and Their Potential for Wine Production

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Abstract: Novel wine yeast strains have the potential to satisfy customer demand for new sensorial experiences and to ensure that wine producers have strains that can produce wine as efficiently as possible. In this respect, hybrid yeast strains have recently been the subject of intense research, as they are able to combine the favourable characteristics of both parental strains. In this study, two *Saccharomyces* "Kéknyelű" grape juice isolates were identified by species-specific PCR and PCR-RFLP methods and investigated with respect to their wine fermentation potential. Physiological characterization of the isolated strains was performed and included assessment of ethanol, sulphur dioxide, temperature and glucose (osmotic stress) tolerance, killer-toxin production, glucose fermentation ability at 16 °C and 24 °C, and laboratory-scale fermentation using sterile "Kéknyelű" must. Volatile components of the final product were studied by gas chromatography (GC) and mass spectrometry (MS). One isolate was identified as a *S. cerevisiae* × *S. kudriavzevii* hybrid and the other was *S. cerevisiae*. Both strains were characterized by high ethanol, sulphur dioxide and glucose tolerance, and the *S. cerevisiae* strain exhibited the killer phenotype. The hybrid isolate showed good glucose fermentation ability and achieved the lowest residual sugar content in wine. The ester production of the hybrid strain was high compared to the control *S. cerevisiae* starter strain, and this contributed to the fruity aroma of the wine. Both strains have good oenological characteristics, but only the hybrid yeast has the potential for use in wine fermentation.

Keywords: *S. cerevisiae*; *S. kudriavzevii* hybrid yeast; PCR-RFLP; "Kéknyelű" grape juice; volatile component analysis; laboratory-scale fermentation; oenological character

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1. Introduction

Wine making is a complex biological process where different microorganisms generate the final product from grape juice. Fermentation progress and the character of wine can be influenced using selected starter strains rather than relying on the microbes already present in the grape juice must. Starter strains are applied in winemaking to reduce spoilage, avoid risk of slow or incomplete fermentation and make good quality wine using

a standard procedure [1]. In the wine industry one of the main trends is the use of new starter yeasts to create high quality wine. Starter strains are isolated originally from the natural environment and selected based on advantageous technological features, such as high ethanol and glucose (osmotic stress) tolerance, low hydrogen sulphide and foam production, killer activity, and other parameters [2–4]. Autochthonous yeasts contribute to the sensory profile of wine through production of specific volatile compounds such as higher alcohols and esters.

The higher alcohols, ethyl esters and acetate esters are the main aromatic components synthetized by yeast during the fermentation. The esters contribute to fruity character of the wine and these compounds usually occur at low concentrations. A reductive fermentation technique, which is commonly used to reduce oxygen concentrations and lower temperatures increase the production of esters. Other components, such as sugars, alcohols and organic acids, etc. also influence the organoleptic character of the wine [5]. Ethanol is one of the main components of the wines, its concentration is usually between 7% and 17%. Ethanol level is dependent on the sugar content of the grape juice and the fermentation capacity of the starter strain. Some of the organic acids, for example tartaric acid, malic acid and citric acid, originate from the grape [6]. The concentration of these components is influenced by the grape variety and the weather conditions. The volatile acidity of wine is usually between 10–15% of the total acids. Acetic acid accounts for 90% of the total volatile acidity, and at high concentrations (>1.2 g/L) has an undesirable effects on wine quality due to it distinctive smell and taste of vinegar [5].

The most commonly used pure starter culture is *Saccharomyces cerevisiae*, but there are some commercially available products containing other species (both *Saccharomyces* and non-*Saccharomyces* species). Of particular interest are hybrid yeasts, which potentially combine desirable features of both parental species. Furthermore, some hybrid yeasts can adapt better to the changing conditions and show beneficial technical parameters in wine fermentation relative to parental strains. Examples are greater cold tolerance and higher glycerol production [7–9]. Application of hybrid strains may enrich the aroma complexity of wine, something which otherwise might necessitate the use of two or more yeasts during fermentation [10]. According to Gamero and colleagues [11], interspecific hybrids can release more flavour compounds from grape aroma precursors than non-hybrid strains.

Hybrid yeast can either be produced in the laboratory using different methods [12,13] or isolated from natural sources. Artificial generation of new hybrid strains with beneficial properties is a widely used strategy based on application of well-characterized and selected parental strains [14–17]. This process has resulted in potentially useful offspring strains with advantageous properties for fermentation [15]. Hybrid strains belonging to the *Saccharomyces* genus have been isolated from different alcoholic beverages including beer, cider and wine [18–20]. Intra- or inter-specific hybridization increases genetic diversity and, as a consequence, hybrid strains can adapt better to changes in the fermentation process [18,21,22]. Taxonomically related species from *Saccharomyces* genus can generate stable hybrids [23], but these natural interspecies hybrids usually cannot produce viable spores due to a post-zygotic barrier [24]. In this respect, a good example is the lager brewing yeast *Saccharomyces pastorianus*, which is a hybrid of *S. cerevisiae* and *S. eubayanus* [22,24–26].

In this study, two previously isolated *Saccharomyces* strains were identified using species-specific PCR and PCR-RFLP methods, including 3 different genes (*CYR1*, *HIS4*, YCL008c). The isolated yeasts were characterized physiologically and applied in laboratory-scale fermentation. Our aim was to identify taxonomic status of the isolates, study the potential hybrid character of the strains, describe oenologically relevant features and analyse the volatile compounds of the final products. In this study we focused on the development of terroir and hybrid starter yeasts for wine production of the local grape variety “Kéknyelű” grown in the Badacsony wine region of Hungary.

2. Materials and Methods

2.1. Yeast Strains Used in This Study

Yeasts strains were previously isolated from the grape juice of the ancient and local Hungarian grape variety "Kéknyelű" (for example, [27]) on YEPD media (1% yeast extract, 2% peptone, and 2% dextrose, 1.5% agar) which were supplemented with antibiotics and an antifungal agent to inhibit the growth of moulds and bacteria in the following concentrations: ampicillin (Ap; Duchefa, Haarlem, The Netherlands) 150 µg/mL, chloramphenicol (Cm; Duchefa, Haarlem, The Netherlands) 20 µg/mL, biphenyl (Bif; Sigma-Aldrich, St. Louis, MO, USA) 150 µg/mL [28]. These two isolates were selected for further investigation based on results of ITS fragment sequencing. Two-peaked pattern in the sequences was determined in case of the isolates and according to these results, we decided to clarify taxonomic status of the isolates and study the potential hybrid character. Different laboratory strains and commercially available starter strains were used as references. All yeast strains were cultivated on YM medium (0.3% yeast extract, 0.7% peptone, 0.3% malt extract, 1% glucose, 2% agar) at 30 °C. Table 1 shows all investigated strains and their origin.

Table 1. A list of strains used in this study.

Strains Designation	Species	Source	Reference
H1	potential *Saccharomyces cerevisiae*	"Kéknyelű" must, Badacsonytomaj, Hungary	this work
H2	potential *S. cerevisiae* × *S. kudriavzevii* natural hybrid	"Kéknyelű" must, Badacsonytomaj, Hungary	this work
ST	Fermol Elegance *S. cerevisiae* commercially available starter	AEB Hungária Kft.	AEB Hungária Kft.
Sb	*Saccharomyces bayanus* * DBVPG 8001	University of Debrecen, Department of Genetics and Applied Microbiology Culture Collection	-
Sc	*Saccharomyces cerevisiae* CBS 1171	National Collection of Agricultural and Industrial Microorganisms MATE	Meyen ex E. C. Hansen, 1883
Seu	*Saccharomyces eubayanus* CBS 12357	VTT Culture Collection	[29]
Skud	*Saccharomyces kudriavzevii* CBS 8840	VTT Culture Collection	[30]
Spar	*Saccharomyces paradoxus* CBS 432	VTT Culture Collection	[31]
Spas	*Saccharomyces pastorianus* VTT-A63015	VTT Culture Collection	[32]
Su	*Saccharomyces uvarum* CBS 395	University of Debrecen, Department of Genetics and Applied Microbiology Culture Collection	[33]
S6	*Saccharomyces cerevisiae* K2 killer sensitive strain	National Collection of Agricultural and Industrial Microorganisms MATE	-

* characterized as multiple hybrids between *Saccharomyces uvarum* (controversially classified as *S. bayanus var. uvarum*), *S. cerevisiae* and *S. eubayanus* [34].

2.2. Species Specific PCR and PCR-RFLP Analyses of CYR1, HIS4, YCL008c Genes

A NucleoSpin DNA Purification kit was used for DNA isolation according to the manufacturer's protocol. Genomic DNA was applied as template for PCR reactions.

The amplification of species-specific fragments was carried out separately in a total volume 20 µL using 10 µL 2× Phusion high fidelity PCR mix (ThermoFisher Scientific, Waltham, MA, USA), 1 µL (10 µM) of each primer (Table 2) [35], 7 µL nuclease-free water and 1 µL genomic DNA as template. The following temperature settings were used: 30 s

at 98 °C, followed by 35 cycles of 10 s at 98 °C, 45 s at 65 °C, 1 min at 72 °C, and a final extension step of 5 min at 72 °C. PCR fragments were separated with electrophoresis (2% agarose).

The *CYR1*, *HIS4* and *YCL008c* genes and restriction enzymes were selected based on the suggestions of Gonzalez and colleagues [20] and Casaregola and colleagues [36]. The primers listed in Table 2 were used in the RFLP analysis. PCR amplification of *CYR1* gene was carried out in a total volume of 20 µL consisting of 10 µL 2× Phusion high fidelity PCR mix (ThermoFisher Scientific, Waltham, MA, USA), 1 µL (10 µM) of each primer, 7 µL nuclease-free water and 1 µL genomic DNA as template. The mixture was subjected to an initial denaturation step of 30 s at 98 °C, followed by 35 cycles consisting of 10 s at 98 °C, 30 s at 60 °C and 1 min at 72 °C, and a final extension step of 5 min at 72 °C. Amplification of the *HIS4* and the *YL008c* genes was performed in a mixture containing: 20 µL 2× Dreamtaq master mix (ThermoFisher Scientific, Waltham, MA, USA), 2 µL (10 µM) of each primer, 14 µL nuclease-free water and 2 µL genomic DNA as template. The following temperature settings were used: 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 46 °C (HIS4) or 55 °C (YCL008c) and 1 min at 72 °C, and a final extension step of 7 min at 72 °C. PCR product of *CYR1* was digested with *Hae*III and *Msp*I restriction enzymes, *Hind*III and *Eco*RV enzymes were used for *HIS4* DNA fragment digestion, while *Pst*I and *Eco*RV enzymes were used for *YCL008c* PCR fragment digestion in the following reaction mix: 2 µL appropriate buffer 5×, 0.5 µL enzyme, 15.5 µL nuclease-free water and 2 µL PCR products. Incubation time was 60 min at 37 °C. Digested fragments were separated on 2% agarose gel stained with ethidium-bromide and visualized under UV light.

2.3. Inoculum Preparation and Physiological Assays

Inoculum preparation was performed as follows: yeast strains were grown overnight in YM medium at 30 °C. Two ml aliquots of each sample were harvested by centrifugation at 13,000 rpm for 5 min and the supernatant was removed. The pellet containing yeast cells was washed with sterile 0.8% NaCl solution 3 times to remove components of the medium. The optical density (OD) of yeast cells was measured at 600 nanometers and the density of cell suspension was adjusted to between 0.3–0.4 OD before physiological tests corresponding to approximately 3–4 $\times$ 10^7 cells/mL.

For the ethanol tolerance test, the yeast suspension was plated onto YM agar supplemented with 30 v/v% ethanol and incubated at 30 °C for 48 h. The high ethanol concentration was chosen based on preliminary experiments (data not shown) to represent the differences between the species. After 2 days of incubation, plates were checked for the presence of yeast colonies.

For the glucose (osmotic stress) tolerance test, the isolates were plated on YM agar in the presence of 30% glucose and the samples were incubated at 30 °C for 48 h. Tolerant strains were capable of growing after the incubation.

For determination of sulphur dioxide tolerance, a microdilution method was used. The different strains were inoculated in the wells of the microtiter plate (1 $\times$ 10^5 CFU) containing YM liquid medium (adjusted to pH 3.50 with citric acid), and incubated at 30 °C for 72 h. Potassium metabisulphite was used to provide sulphur dioxide in the medium, and the final concentrations ranged from 0 to 300 mg/L. The minimal inhibitory concentration was determined as the lowest concentration of potassium metabisulphite that completely inhibits growth of the organism in the microdilution wells.

For the glucose fermentation test the isolates were inoculated into Durham tubes containing phenol purple broth medium (1% glucose, 2% yeast extract, 0.0016% bromophenol purple indicator) and incubated at 16 °C and 24 °C for 7 days. Glucose-free medium was used as a negative control. The change of colour and gas (CO_2) production in the Durham tubes indicated fermentation ability of isolates [37].

For the temperature tolerance test, YM plates were used to test temperature tolerance of samples. Yeasts were incubated at 5 °C, 16 °C, 24 °C and 37 °C and monitored after 5 days.

Table 2. Sequences of applied primer pairs, annealing temperature and fragment size of amplified region.

Analyzes	Genus/Species	Name of Primers	Primer Sequences	Tm (°C)	Fragment Size (bp)	Reference
Species—specific PCR	*S. paradoxus*	Spar F7 (forward) Spar R7 (reverse)	CTTTCTACCCCTTCTCCATGTTGG CAATTTCAGGGCGTTGTCCAACAG	66	739	[35]
	S. cerevisiae	ScerF2 (forward) ScerR2 (reverse)	GCGCTTTACATTCAGATCCCGAG TAAGTTGGTTGTCAGCAAGATTG	63	149	
	S. bayanus/S. uvarum	SbayF1 (forward) SbayR1 (reverse)	GCTGACTGCTGCTGCTGCCCCCG TGTTATGAGTACTTGGTTTGTCG	62	275	
	S. kudriavzevii	SkudF2 (forward) SkudR1 (reverse)	ATCTATAACAAACCGCCAAGGGAG CGTAACCTACCTATATGAGGGCCT	66	660	
CYR1 gene amplification	*Saccharomyces*	CYR1-5 (forward) CYR1-3 (reverse)	CTACGAAGGAAAGTGTCCTCTTTRGTTCGTGG CCGTGTGTAGAATTTAGTGTAGAATTGACRGC	60	570	[20]
HIS4 gene amplification	*Saccharomyces*	HIS4-U (forward) HIS4-L (reverse)	ACTCTAATAGTGACTCCG AACTTGGGAGTCAATACC	46	2100	[36]
YCL008c gene amplification	*Saccharomyces*	YCL008c-U (forward) YCL008c-L (reverse)	TTCGTTGGATGTGCCATCG GGAGCCACCAAGGGATGG	55	1600	

For the killer toxin production test, K2 killer sensitive *Saccharomyces cerevisiae* strain (S6) was spread on the surface of methylene-blue containing medium [38]. The yeasts tested were inoculated onto the prepared medium and incubated for 3 days at 24 °C. Presence of a clear zone and blue margin around the colony indicated that the yeast strain is a K2 killer toxin producer strain.

2.4. Laboratory-Scale Fermentation and Analysis of Final Products

Fermentations were carried out in triplicate in 250 mL Erlenmeyer flasks with 200 mL of sterile "Kéknyelű" 2016 grape must (22.12 BRIX%). The "Kéknyelű" grape was destemming and crushing mechanically and treated with $K_2S_2O_5$ (50 mg/kg) before it was gently pressed (maximum 1.66 bar) to collect the grape juice. The grape juice was clarified at 8–10 °C for 12 h. Clear liquid was collected and delivered to the laboratory and frozen until use. Before the fermentation process, the must was filtered. 100 µL of prepared yeast suspension corresponding to approximately 3–4 × 10^7 cells/mL (see Section 2.3) were used for inoculation under anaerobic conditions, achieved using an airlock containing sterile paraffin oil. Vinification was monitored by measuring weight loss in every 24 h at 16 °C, and the fermentation was stopped after 480 h (20 days), when the weight loss was minimal (approximately 0.25 g in 24 h). After the 20-day fermentation, viability of the strains was measured using LunaII automated cell counter (CEBIOSYS, Budapest, Hungary) and pH, residual sugar content, ethanol and volatile acid concentrations of final products were analysed (NARIC-Viticulture and Oenology Institute, Badacsonytomaj, Hungary). NIR spectrometry was used for detection of ethanol concentration (*v*/*v*%), residual sugar content was determined by the Schrool method [39], total volatile acidity and pH were analysed by classical analytical methods. Analytical measurements were carried out by an accredited laboratory (Badacsony Wine and Viticulture Testing Laboratory, Badacsonytomaj, Hungary; Registration number in Hungary: NAH-1-1496/2019). The accreditation included the mandatory use of international standards.

2.5. Identification of Volatile Compounds by Gas Chromatography-Mass Spectrometry (GC-MS) Method

The volatile compounds of the samples were identified based on the method developed by Torrens et al. [40]. The GC-MS was equipped with an AOC-6000 autosampler (Shimadzu, Kyoto, Japan) applied in SPME mode throughout the measurements using a 1 cm 50/30 µm DVB/CAR/PDMS SPME fibre (Supelco, Bellefonte, PA 16823, USA), 20-mL sample vials with magnetic crimp caps and Teflon-lined septa. For the analysis, the method described by Cai et al. [41] was applied with minor modifications. According to this, each sample vial contained 1.2 g NaCl, 4.0 mL sample (either wine or aqueous ethanol (12 *v*/*v*%) for the standards), and 50 µL of internal standard solution (3-octanol, Sigma-Aldrich, Budapest, Hungary), 200 µg/mL in 12% aqueous ethanol). The autosampler program was set as follows: incubation of sample at 40 °C for 20 min and extraction at 40 °C for 20 min with agitation at 500 rpm, analysis desorption time was 2 min in the Split/Splitless injector of the GC-MS. The SPME fibre was then inserted into the condition port heated to 260 °C for 10 min to avoid carryover between runs by ensuring full desorption of all analytes from the fibre.

The samples were analysed using a GCMS-QP2020 gas chromatograph coupled to a single quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Chromatographic separations were carried out using a Trace Gold, TG-WaxMS capillary column with the following characteristics: 60 m, 0.25 mm ID × 0.25 µm film thickness, 100% polyethylene glycol (ThermoFischer Scientific, Waltham, MA 02451, USA). The carrier gas was helium at a flow rate of 2 mL/min. The column oven temperature program was: initial temperature 45 °C for 0 min, 45–120 °C at a rate of 3 °C/min held for 10 min, 120–165 °C at a rate of 3 °C/min and held for 10 min, and 165–230 °C at a rate of 3 °C/min and held for 17 min. The total run time was 100.67 min. An electron ionization source was used, with a source temperature of 230 °C and an electron energy of 70 eV. Mass spectral data were collected over the

range of 40–400 m/z in the full scan mode (scan time 0.5 s). Data were acquired using the GC-MS solution ver. 4.45 software. The volatile compounds were identified and quantified using standard reference compound of ethyl-acetate (4.0 min), isobutyl acetate (6.1 min), ethyl butyrate (6.4 min), isobutanol (7.9 min), isopentyl acetate (8.9 min), butanol (9.6 min), isoamyl-alcohol (11.8 min), ethyl hexanoate (12.8 min), hexyl acetate (14.3 min), ethyl lactate (17.1 min), 1-hexanol (17.6 min), acetic acid (21.9 min), linalool (25.5 min), butyric acid (29.5 min), diethyl succinate (32.2 min), α-terpineol (33.5 min), β-citronellol (39.1 min), 2-phenethyl acetate (41.4 min), geraniol (43.6 min) and 2-phenylethanol (46.6 min). The R^2 values of the calibration curves were above 0.99 for each compound. The concentrations of volatiles were expressed as mg/L.

2.6. Measurement of the Glycerol Content

For the glycerol measurement, 20 µL of each wine was directly injected onto an ion exchange column without any sample pre-treatment [42]. A modular HPLC system (Shimadzu, Kyoto, Japan) equipped with an SCL-10AVP system controller, LC10-ADVp pump, DGU-14A degasser, SIL10-ADvp autosampler, CTO-10ASvp column oven and a RID 10A detector as well as a Hi-Plex H (Agilent, Santa Clara, CA 95051, USA), 7.7 × 300 mm, 8 µm column was used for the analysis. The isocratic mobile phase was 0.004 M sulphuric acid at a flowrate of 0.4 l L/min, while the column was maintained at 75 °C. For the instrument control, data acquisition and evaluation, the Class VP ver. 6.2 software was applied, where the glycerol eluted at 22 min and the R^2 values of the calibration curve was 0.9996.

2.7. Statistical Analysis of Physiological Assays and GC-MS Data

All experiments were performed in triplicate and the results were presented as the average value with standard deviation. The concentration data of volatile compounds and glycerol were analysed using Methabo Analyst ver. 5.0 (Ottawa, Canada). The applied data was not normalized, transformed and scaled. Significant differences among the samples were calculated using the ANOVA test, the *P*-value cut-off was 0.05. Tukey post-hoc analysis was performed for physiological test data and Fisher's LSD post-hoc analysis was applied for GC-MS data.

3. Results

3.1. Molecular Identification and Confirmation of Hybrid Status

In this study, two yeasts (H1 and H2) were selected among the 480 isolates from Hungarian "Kéknyelű" grape juice on YEPD medium containing antibiotics (chloramphenicol and ampicillin) and the antifungal agent biphenyl [28].

Sequencing of the ITS region of the H1 and H2 strains revealed the presence of more than one peak in the chromatograms at the same position (results not shown). Repeated multiple sequencing of the ITS region from single colonies also resulted in the same two-peaked pattern in the sequences. This result can be explained by allelic variations of ITS sequences in diploid cells or by the presence of gene copies derived from different strains. A similar result was published where it was described that it has not been possible to clearly identify the ITS sequence of a *S. cerevisiae* × *S. kudriavzevii* hybrid isolated from Austria [43]. Consequently, we decided to use molecular identification methods to clarify taxonomic status of our isolates. Species-specific PCR and PCR-RFLP on three different genes (*CYR1*, *HIS4*, *YCL008c*) were carried out (see Section 2.2) for molecular identification of the two potential hybrid isolates.

3.1.1. Species-Specific PCR

Four species-specific primer pairs (Table 2) [35] were used for the identification of the isolated strains. According to our results, the expected fragment size was obtained for all reference strains (*S. paradoxus* 739 bp, *S. bayanus* 275 bp, *S. cerevisiae* 150 bp, *S. kudriavzevii* 660 bp). In the *S. paradoxus* and *S. kudriavzevii* specific reaction we were not able to detect

amplicons in any samples, with the exception of the reference strain (Figure 1). Non-specific fragments were observed in the case of H1, H2 and the starter strain for the *S. bayanus*-specific reaction. Figure 1 illustrates that H1, H2, Sc and ST strains produced 150 bp long fragments using *S. cerevisiae* specific primers. The restriction pattern analyses of *CYR1*, *HIS4*, *YCL008c* genes were used for further analysis of H1 and H2 isolates.

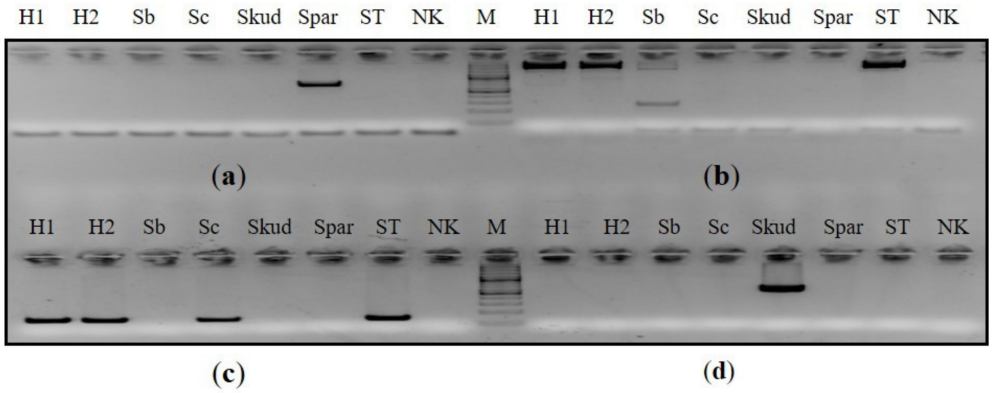


Figure 1. The results of species-specific PCR: (**a**) amplification with SparF7 and SparR7 primers, specific for *S. paradoxus*; (**b**) amplification with SbayF1 and SbayR1 primers, specific for *S. bayanus*; (**c**) amplification with ScerF2 and ScerR2 primers, specific for *S. cerevisiae*; (**d**) amplification with SkudF2 and SkudR1 primers, specific for *S. kudriavzevii*. H1, H2: isolates investigated, Sb: *S. bayanus* DBVPG 8001, Sc: *S. cerevisiae* CBS 1171, Skud: *S. kudriavzevii* CBS 8840, Spar: *S. paradoxus* CBS 432, ST: Fermol Elegance *S. cerevisiae* starter strain, NC: negative control containing water instead of DNA, M: molecular weight standard, Gene ruler 100 bp (ThermoFisher Scientific, Waltham, MA, USA). The figure was constructed based on different photos of the electrophoresis.

3.1.2. Restriction Pattern Analysis

Amplification of the *CYR1* gene produced a 560 bp fragment for every strain. *Hae*III digestion was not observable in the case of the H1 isolate and the starter (ST) *S. cerevisiae* strain (Figure 2a), but after digestion with *Msp*I enzyme 380 bp and 180 bp size fragments were detected (Figure 2b). In the case of the *S. kudriavzevii* reference strain, *Hae*III digestion of the *CYR1* gene resulted in 260 bp, 160 bp and 140 bp PCR fragments, while the *Msp*I enzyme treatment produced 420 bp and 140 bp fragments. In the case of *S. paradoxus* the same product sizes were detected (380 bp and 180 bp) after digestion with *Hae*III and *Msp*I enzymes. As shown in Figure 2, the H2 strain can be characterised by a mixed restriction pattern of *S. cerevisiae* and *S. kudriavzevii* using *Hae*III and *Msp*I enzymes, but in the case of the H1 isolate only the *S. cerevisiae* characteristic pattern was observable.

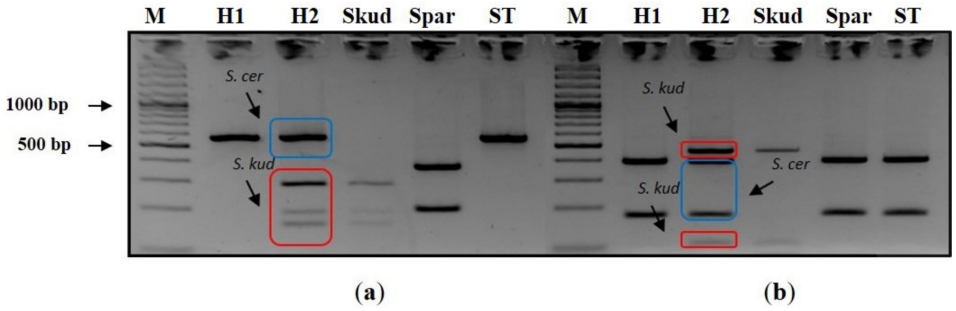


Figure 2. Patterns of *CYR1* PCR fragment after digestion with *Hae*III (**a**) and *Msp*I (**b**) restriction enzymes, H1, H2: isolates investigated, Skud: *S. kudriavzevii* CBS 8840, Spar: *S. paradoxus* CBS 432, ST: Fermol Elegance *S. cerevisiae* starter strain, M: molecular weight standard, Gene ruler 100 bp (ThermoFisher Scientific, Waltham, MA, USA). Blue and red rectangles represent *S. cerevisiae* and: *S. kudriavzevii* specific fragments, respectively. The figure was constructed based on different photos of electrophoresis.

Digestion of the *HIS4* gene was carried out with *Hind*III and *Eco*RV enzymes separately. As shown in Figure 3a, *S. bayanus*, *S. eubayanus* and *S. uvarum* strains did not contain *Hind*III restriction sites. *S. cerevisiae*, *S. paradoxus* reference strains and H1 showed the same restriction pattern (800 bp, 700 bp, 600 bp). The natural hybrid *S. pastorianus* (*S. cerevisiae* × *S. bayanus*) can be characterized with 2100 bp, 800 bp, 700 bp and 600 bp long fragments. In the case of *S. kudriavzevii*, 1300 bp and 800 bp fragments were detected after *Hind*III digestion. The results of the digestion patterns of the reference strains correlate with that seen in the literature [36]. The H2 isolate can be characterized by 1300 bp, 800 bp, 700 bp and 600 bp fragments, while H1 can be characterized by 800 bp, 700 bp and 600 bp fragments. Using *Eco*RV enzyme for *HIS4* gene digestion, a 2100 bp fragment was detected in the case of *S. cerevisiae*, *S. paradoxus*, *S. uvarum* and *S. bayanus* reference strains (Figure 3b). Digestion of the PCR fragment of the *S. pastorianus* reference strain resulted in 2100 bp, 1700 bp and 400 bp products, as can be seen in Figure 3b. The PCR fragments of the *S. kudriavzevii* and *S. eubayanus* reference strains contained one *Eco*RV restriction site in the *HIS4* gene, which resulted in 900 bp and 1200 bp fragments (*S. kudriavzevii*) and 400 bp and 1700 bp fragments (*S. eubayanus*). Digestion of the H1 sample with *Eco*RV was not apparent. The H2 strain can be characterized after *Eco*RV treatment by 2100 bp, 1200 bp and 900 bp products. This was believed to be a mixture of the *S. kudriavzevii* (1200 bp and 900 bp) and *S. cerevisiae* (2100 bp) patterns. However, there was a 700 bp DNA fragment of the H2 sample, which appeared consistently with this strain only, but sequencing the isolated 700 bp fragment, revealed this to be a non-specific amplicon.

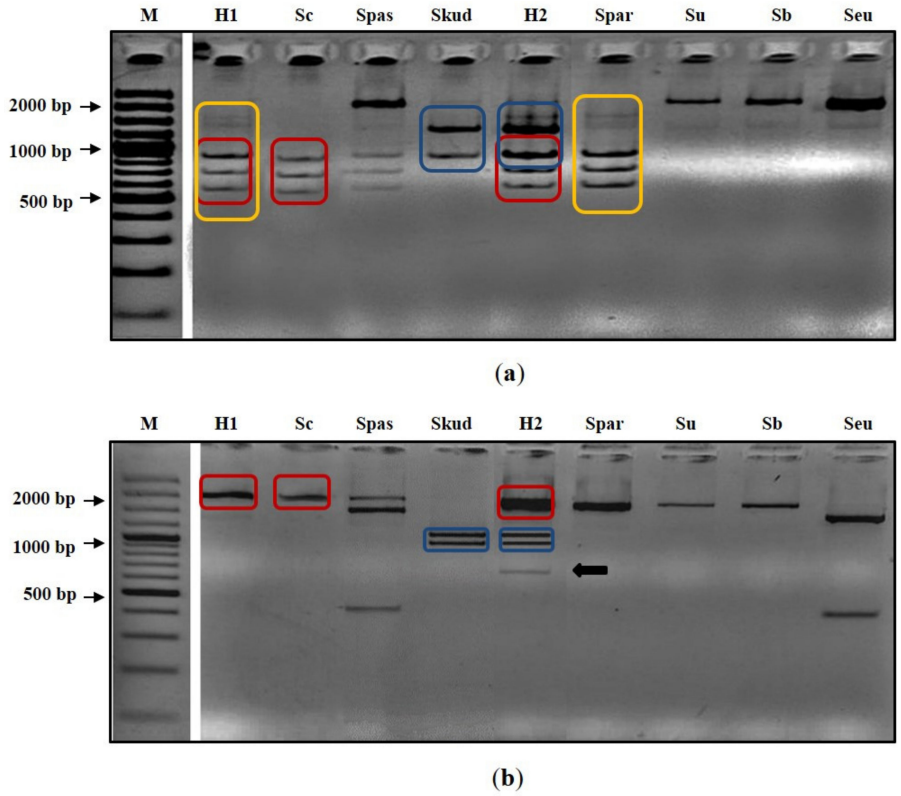


Figure 3. Patterns of *HIS4* PCR fragment after digestion with *Hind*III (**a**) and *Ecor*V (**b**) restriction enzymes, H1, H2: isolates investigated, Sb: *S. bayanus* DBVPG 8001, Sc: *S. cerevisiae* CBS 1171, Skud: *S. kudriavzevii* CBS 8840, Spar: *S. paradoxus* CBS 432, Spas: *S. pastorianus* VTT-A63015, Su: *S. uvarum* CBS 395, Seu: *S. eubayanus* CBS 12357, M: molecular weight standard, Gene ruler 100 bp (ThermoFisher Scientific, Waltham, MA, USA). The Figure was constructed based on different photos of the electrophoresis. Blue and red rectangles represent *S. cerevisiae* and *S. kudriavzevii* specific fragments, respectively. The yellow rectangle represents the *S. paradoxus* pattern, the black arrow represents non-specific fragment.

Amplification of *YCL008c* resulted in a 1600 bp DNA product, which was subsequently digested with *Eco*RV and *Pst*I enzymes (Figure 4). According to the results the following species did not contain *Eco*RV or *Pst*I restriction sites: *S. paradoxus*, *S. uvarum*, *S. bayanus*. *S. eubayanus* (*Eco*RV) and *S. cerevisiae*, *S. kudriavzevii*, *S. paradoxus* (*Pst*I). *S. cerevisiae* and *S. kudriavzevii* can be characterized by 2 different fragments (1100 bp and 500 bp), while *S. pastorianus* showed a mixed pattern of *S. cerevisiae* and *S. bayanus/eubayanus/uvarum* (1100 bp and 500 bp, 1600 bp, respectively) after *Eco*RV digestion (Figure 4a). It should be noted that the mixed nature of *S. pastorianus* can be explained by the fact that *S. pastorianus* is a natural hybrid of *S. cerevisiae* and *S. eubayanus*. *Eco*RV digestion of H1 and H2 samples produced the same pattern seen for *S. cerevisiae* and *S. kudriavzevii*. Using the *Pst*I enzyme, two different patterns were detectable, one of them contained 1150 bp, 300 bp and 150 bp fragments (*S. bayanus*, *S. eubayanus*, *S. uvarum*), while the other contained 1600 bp, 1150 bp, 300 bp and 150 bp fragments (*S. pastorianus*). Digestion was not detectable using the *Pst*I enzyme in case of H1 and H2 strains (Figure 4b).

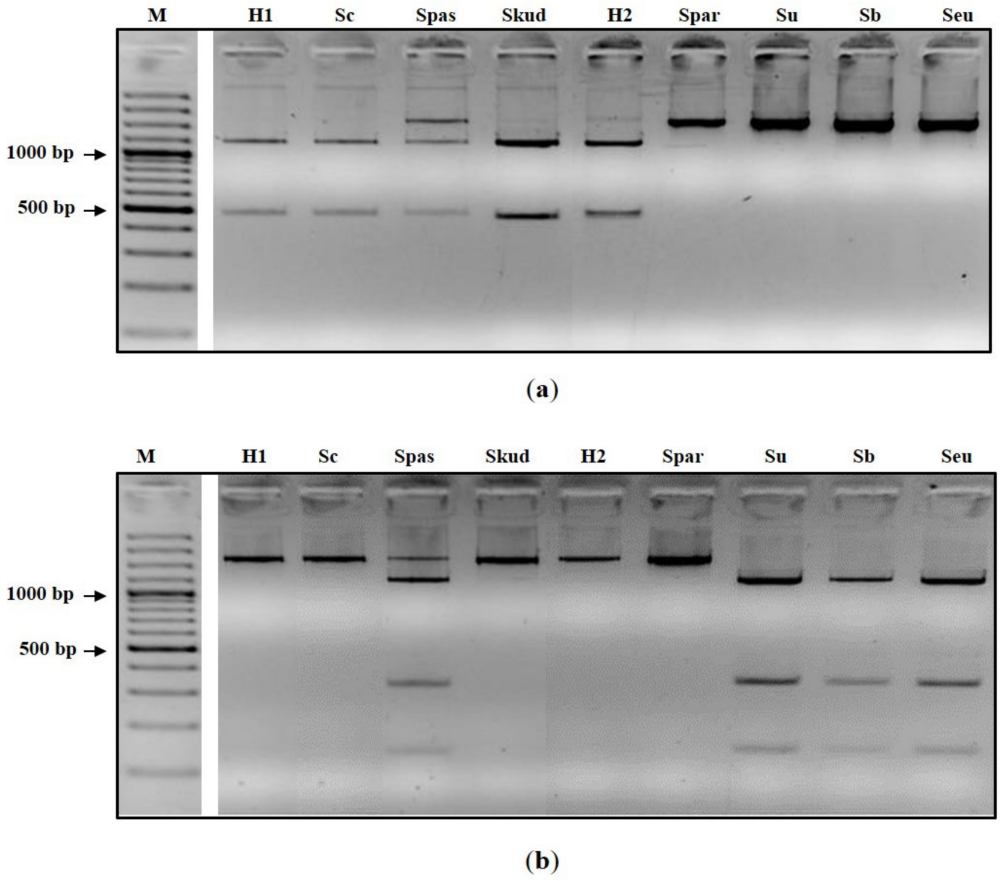


Figure 4. Patterns of *HIS4* PCR fragments after digestion with *Ecor*V (**a**) and *Pst*I (**b**) restriction enzymes, H1, H2: isolates investigated, Sb: *S. bayanus* DBVPG 8001, Sc: *S. cerevisiae* CBS 1171, Skud: *S. kudriavzevii* CBS 8840, Spar: *S. paradoxus* CBS 432, Spas: *S. pastorianus* VTT-A63015, Su: *S. uvarum* CBS 395, Seu: *S. eubayanus* CBS 12357, M: molecular weight standard, Gene ruler 100 bp (ThermoFisher Scientific). Figure was constructed based on different photos of electrophoresis.

3.2. Oenological Features of Investigated Strains

Plating assays (see Section 2.3) were used for testing the different tolerances of H1, H2, and relevant reference strains regarding important characteristics in winemaking, such as osmotic, ethanol, sulphur dioxide, temperature stresses and killer toxin production. Results of oenological investigations are shown in Table 3 and photos are presented in Supplementary Figures S1 and S2. Growth ability of investigated yeasts was similar at 5 °C; none were able to grow at this temperature. In this test, 24 °C was the control temperature,

which was ideal for cultivation of every strain, however, the *S. cerevisiae* CBS 1171 strain showed weaker growth. The *S. pastorianus* and *S. paradoxus* reference strains could grow better at 16 °C than the two S. *cerevisiae* and the *S. kudriavzevii* reference strains. The H1 and H2 strains showed medium growth at 16 °C. The *S. cerevisiae* CBS 1171 reference strain and H2 hybrid were characterised by medium tolerance at 37 °C. The *S. cerevisiae* Fermol Elegance starter strain and the H1 isolate could grow better at 37 °C, in contrast to the *S. paradoxus* strain, which had limited growth at this temperature. The *S. paradoxus* and *S. kudriavzevii* strains were not able to grow at 37 °C. The temperature tolerance of the H2 isolates at 37 °C resembled the growth ability of *S. cerevisiae* CBS1171.

Table 3. Oenological characterization of investigated strains in the presence of 30% glucose and 30% ethanol, sulphur dioxide tolerance after 48 and 72 h, temperature tolerance at 5 °C, 16 °C, 24 °C and 37 °C, glucose fermentation ability was determined at two different temperatures (16 °C, 24 °C). Killer activity was monitored at 24 °C.

Strains	Tolerance to				Glucose Fermentation		Killer Activity	Temperature Tolerance		
	Glucose 30%	Ethanol 30%	Sulphur Dioxide 30 °C (mg/L)							
	30 °C	30 °C	48 h	72 h	16 °C	24 °C	24 °C	16 °C	24 °C	37 °C
H1	+++	+++	240	240	3	2	+++	++	+++	+++
H2	++	+++	200	200	3	2	-	++	+++	++
ST	+++	+++	240	260	3	2	-	+	+++	+++
Sc	++	++	60	80	4	3	-	+	++	++
Spar	+++	+++	160	160	3	2	-	+++	+++	+
Skud	++	+	40	60	3	2	-	+	+++	-
Spas	nd	++	60	80	3	2	-	++	+++	-

H1, H2: isolates investigated, Sc: *S. cerevisiae* CBS1171, Skud: *S. kudriavzevii* CBS 8840, Spar: *S paradoxus* CBS 432, Spas: *S. pastorianus* VTT-A63015, ST: *S. cerevisiae* Fermol Elegance starter strain, nd: not determinate, -: no growth/activity, +: low growth/activity, ++: medium growth/activity, +++: high growth/activity, 2, 3, 4: number of days before strains can start fermentation. None of the strains was able to grow at 5 °C. Results are averages of three parallel experiments.

Glucose fermentation ability of investigated strains was monitored at two temperatures (16 °C and 24 °C, see Table 3). Glucose fermentation of the strains in most cases was detected after 2 days at 24 °C and after 3 days at 16 °C. In the fermentation tests, the *S. cerevisiae* CBS 1171 showed slower fermentation ability at 16 °C and 24 °C than the H1 and H2 isolates and the other reference strains. Each strain began fermenting one day later at 16 °C compared to 24 °C. The H2 isolate exhibited at least the same glucose fermentation ability as the representative potential parental species in this test.

High glucose and ethanol tolerance of industrial yeasts are important properties because starter strains are exposed to these stressors during the fermentation process. According to our investigations, all the nine tested yeasts strains could tolerate 30% glucose concentration (Supplementary Figure S1), though the H2 hybrid yeast showed less robust growth, compared to the *S. cerevisiae* CBS 1171 and *S. kudriavzevii* CBS 8840 representing the potential parental species. The H1 isolate, Fermol starter strain, and *S. paradoxus* reference yeast showed good growth ability in the presence of 30% glucose. All of the strains were able to grow in the presence of 30% ethanol; however, the *S. kudriavzevii* and *S. cerevisiae* CBS 1171 reference strains showed lower tolerance. The *S. cerevisiae* × *S. kudriavzevii* (H2) hybrid can be characterized by high ethanol tolerance despite the *S. kudriavzevii* reference strain showing decreased tolerance in the presence of 30% ethanol. The H1 strain also exhibited high ethanol tolerance comparable to the *S. cerevisiae* Fermol starter.

Sulphite is widely used in winemaking as an antimicrobial agent and antioxidant. Examining the sulphite tolerance of H1, H2 and the reference strains, we found (Table 3 and Supplementary Figure S2) that H1 and H2 isolates as well as the commercial starter (ST) showed significant tolerance to sulphite, the minimal inhibitory concentration (MIC) was found to be equal or above 200 mg/L. *S. paradoxus* strain had higher tolerance as well

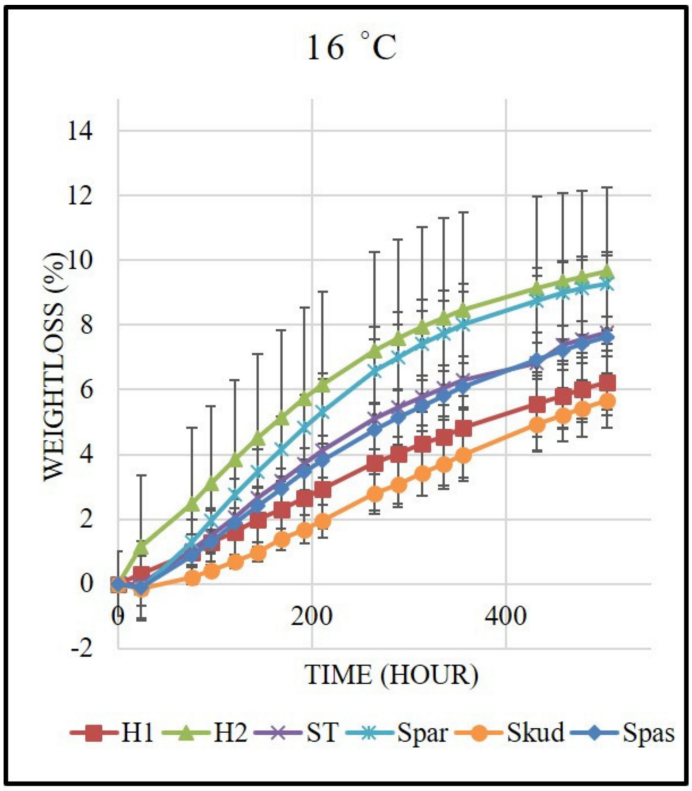
(160 mg/L). This is consistent with the observation that the minimal inhibitory concentration of potassium metabisulphite, generally used to produce sulphite in wine, was reported 200 mg/L in the case of *S. cerevisiae* [44]. Other reference strains (*S. cerevisiae* CBS1171, Skud: *S. kudriavzevii* CBS 8840, *S. pastorianus* VTT-A63015,) was inhibited with 40–60 mg/L potassium metabisulphite after 48 h of incubation. In this case the MIC values at 48 and 72 h were either the same or only slightly different.

Killer starter strain use in fermentation is an effective way to enhance dominance of the applied killer yeast against the natural yeast community of non-sterile must. In this study, K2 killer properties of all strains were investigated and we detected killer toxin production in the case of the H1 isolate only, but with very high activity.

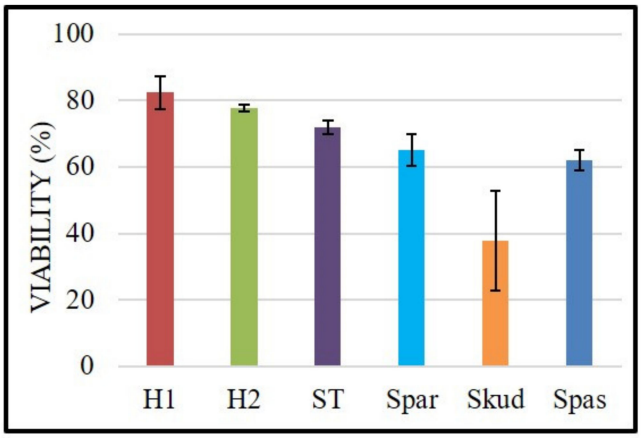
3.3. Kinetics of "Kéknyelű" Fermentation and Analytical Parameters of Final Products

Vinification properties of yeast strains were monitored using CO_2 weight loss measurement, which is a simple method to detect progress of fermentation. The fermentation capacity of the investigated strains (H1 and H2, *S. paradoxus* CBS 432, *S. kudriavzevii* CBS 8840, *S. pastorianus* VTT-A63015, Fermol Elegance *S. cerevisiae* starter) was monitored over 480 h (20 days) at 16 °C. After fermentation, pH, residual sugar contents and volatile acid concentrations of final products were analysed and the results are presented in Figure 5. According to our results, pH values of investigated samples were very similar and ranged between pH 3.32 and 3.76. In contrast, residual sugar concentrations of the wine showed large variance. The wine made by the *S. kudriavzevii* strain had the highest residual sugar concentration (average 58.87 ± 3.37 g/L), indicating that the fermentation was not complete. We could detect also high concentration (45.1 ± 1.01 g/L) in case of H1 strain. Wine produced by the H2 hybrid yeast and *S. paradoxus* reference strain contained less sugar (H2: 4.2 ± 1.88 and Spar: 12 ± 5.16 g/L) compared to the wine made by the other yeasts. Differences in the residual sugar content were observed between H1-H2, ST-H2, Skud-H2, ST-Spas, Skud-Spas, Spar-ST and Skud-Spar strains. Differences were statistically significant as determined by ANOVA analyses (Supplementary Table S1). Residual sugar content of the wine made by H2 strain differed significantly compared to the wines made by potential parental species (ST and Skud). We could detect sugar concentration under 9 g/L in the case of the H2 hybrid isolate (Figure 5c), which is typical for dry wine. The residual sugar content (47.9 ± 12.77 g/L) of the wine made by the control starter strain (ST) was relatively high, as was the concentration of volatile acid (0.67 ± 0.22 g/L). However, H1 and H2 produced the most volatile acid, (0.73 ± 0.04 and 0.8 ± 0.19 g/L). H2 fermented wine showed significantly higher concentration of volatile acidity compared to Spar and Spas strains (Supplementary Table S1). The wine made by the *S. pastorianus* reference strain exhibited the lowest concentration of volatile acid (0.14 ± 0.06) among the wine samples investigated (Figure 5c).

In the fermentation capacity test, H2 hybrid and H1 isolate were able to start fermentation already on the first day, exhibiting a short lag phase, in contrast to the other strains, which started the fermentation after 24 h only. As shown in Figure 5a the weight loss of all samples was slow and the intensity varied. The total weight loss values ranged from 5.67% (*S. kudriavzevii*) to 9.65% (H2 hybrid). The fermentation kinetics of the H2 hybrid were similar to the *S. paradoxus* representative strain and the kinetics of the H1 isolate were similar to the *S. kudriavzevii* strain. The investigated yeasts could not complete fermentation at 16 °C within the 480 h tested. After 20 days, viability of every strain was calculated using automatic cell counter LunaII. As shown in Figure 5b more than 60% of the yeast cells survived the fermentation conditions. The exception was the *S. kudriavzevii* reference strain, where the lowest viability (37.9%) was detected at the end of the fermentation. The H1 isolate showed impressive growth ability after 20 days, and 82.4% of the yeast cells could tolerate the fermentation conditions, however, the fermentation kinetics of the strain were similar to the *S. kudriavzevii* reference strain.



(a)



(b)

Yeasts	Residual sugar (g/L)	Volatile acid (g/L)	pH
H1	45.1 ± 1.01	0.73 ± 0.04	3.48 ± 0.02
H2	4.2 ± 1.88	0.80 ± 0.19	3.52 ± 0.08
ST	47.9 ± 12.77	0.67 ± 0.22	3.53 ± 0.20
Spar	12.0 ± 5.16	0.21 ± 0.04	3.35 ± 0.03
Skud	58.8 ± 3.37	0.54 ± 0.04	3.33 ± 0.01
Spas	11.9 ± 4.14	0.14 ± 0.06	3.32 ± 0.005

(c)

Figure 5. Fermentation progress (**a**) and viability of the investigated strains at the end of the fermentation (**b**) at 16 °C and analytical parameters of the fermented must (**c**). H1, H2: isolates investigated, Skud: *S. kudriavzevii* CBS 8840, Spar: *S paradoxus* CBS 432, Spas: *S. pastorianus* VTT-A63015, ST: *S. cerevisiae* Fermol Elegance starter strain. Results are averages of three parallel experiments with standard deviations (**b**,**c**).

3.4. Volatile Compounds Analysis of the "Kéknyelű" Wine

Volatile compounds play an essential role in the quality of wine and the applied starter strain used can contribute to this. We investigated four important chemical groups using GC-MS analysis of the laboratory-scale fermentation samples, these were: organic acids, alcohols, esters and terpenes, which are presented in Table 4 and Supplementary Figure S3. The data in Table 4 were subjected to ANOVA analysis, and the analysis showed that most volatile compounds were significantly different from each other based on p-values (Supplementary Table S2).

The concentration of acetic and butanoic acid were monitored in the final products (see Supplementary Table S2). The acetic feature of the samples was characterized based on the acetic acid level. Wine samples produced with the *S. paradoxus* and *S. pastorianus* reference strains contained the two lowest amounts of acetic acid, 33.06 mg/L (*S. pastorianus*) and 44.24 mg/L (*S. paradoxus*). In contrast, the highest concentration of acetic acid (194.19 mg/L) was found in the wine samples prepared with the H2 hybrid yeast. In this case, the samples contained a higher level of acetic acid compared to the two representative strains, *S. kudriavzevii* and *S. cerevisiae*.

Table 4. Average concentrations of the volatile compounds and glycerol using "Kéknyelű" grape juice for fermentation. The RSD (%) values are brackets.

		Spas [1]	Skud [1]	Spar [1]	ST [1]	H1 [1]	H2 [1]
		Means (mg/L)					
acids	acetic acid	33.06 (17.7)	116.94 (6.5)	44.24 (28.6)	122.31 (4.9)	134.91 (4.8)	194.19 (12.0)
	butanoic acid	bdl	bdl	bdl	bdl	bdl	bdl
alcohols	1-hexanol	0.56 (28.7)	0.42 (6.4)	0.47 (2.7)	0.46 (0.1)	0.66 (39.3)	0.43 (0.1)
	2-phenylethanol	17.35 (29.4)	12.58 (21.1)	31.79 (20.1)	6.14 (1.9)	7.43 (15.9)	15.64 (3.6)
	butanol	2.54 (22.5)	0.51 (42.5)	4.41 (20.4)	0.23 (0.1)	0.26 (6.1)	0.76 (0.2)
	isobutanol	7.77 (43.7)	56.38 (23.4)	14.63 (6.4)	6.34 (2.6)	4.93 (28.3)	16.61 (2.0)
	isopentyl alcohol	59.66 (23.6)	59.75 (21.3)	102.57 (4.8)	29.21 (7.9)	25.37 (21.1)	70.29 (15.1)
	glycerol	4800.01 (6.9)	10,319.64 (5.1)	8072.51 (2.4)	7218.24 (322.5)	3482.6 (11.7)	6521.74 (345.6)
esters	2-phenethyl acetate	0.27 (27.2)	0.1 (14.3)	0.29 (13.8)	0.07 (0.1)	0.11 (12.5)	0.23 (0.1)
	diethyl-succinate	0.06 (21.8)	0.04 (16.4)	0.09 (33.4)	0.03 (0.1)	0.04 (23.5)	0.04 (0.1)
	ethyl-acetate	13.73 (38.3)	11.47 (18.8)	20.58 (12.5)	23.1 (1.8)	10.86 (22.1)	31.06 (5.5)
	ethyl-butyrate	0.20 (17.0)	0.06 (35.3)	0.49 (11.3)	0.24 (0.1)	0.06 (6.63)	0.36 (0.1)
	ethyl-hexanoate	0.69 (34.6)	0.15 (19.3)	1.36 (11.1)	0.65 (0.2)	0.26 (28.1)	1.47 (0.2)
	ethyl-lactate	1.48 (26.9)	0.65 (28.6)	1.86 (26.7)	0.27 (0.1)	0.36 (12.8)	1.67 (0.1)
	hexyl-acetate	0.1 (29.7)	bdl	0.08 (2.3)	0.06 (0.1)	0.06 (34.6)	0.1 (0.1)
	isobutyl-acetate	0.02 (24.2)	0.07 (25.4)	0.03 (3.5)	0.04 (0.1)	0.01 (36.9)	0.05 (0.1)
	isopentyl acetate	1.55 (33.95)	0.23 (45.1)	1.54 (0.6)	0.63 (0.1)	0.21 (13.9)	1.79 (0.2)
terpenes	alpha-terpineol	bdl	bdl	bdl	bdl	bdl	bdl
	beta-citronellol	0.09 (1.6)	0.1 (3.6)	0.09 (0.4)	0.09 (0.1)	0.09 (4.2)	0.09 (0.1)
	geraniol	bdl	bdl	bdl	bdl	bdl	bdl
	linalool	0.004 (5.9)	0.006 (40.6)	0.004 (3.5)	0.005 (4.6)	0.007 (8.9)	0.01 (0.1)

[1] bdl-below detection limit, H1, H2: isolates investigated, Skud: *S. kudriavzevii* CBS 8840, Spar: *S paradoxus* CBS 432, Spas: *S. pastorianus* VTT-A63015, ST: *S. cerevisiae* Fermol Elegance starter strain.

Five different higher alcohols were detected in the wine samples. The 1-hexanol level was not significantly influenced by the yeast strains used, but there were differences for the other four alcohols produced by the different yeast strains. Among the higher alcohols, isopentyl alcohol was the highest in all samples, with concentrations ranging from 25.37 to 102.57 mg/L. This compound is characterized by FEMA as having burnt, cocoa, floral, malty aroma notes. It is found in a wide range of naturally occurring sources, including bananas, wasp pheromone, black truffle, etc. It is often found in wines produced by reductive techniques and this aroma substance is favoured by consumers. H2 hybrid isolate produced a similar amount of isopentyl alcohol as *S. kudriavzevii* representing a potential parental species, and twice as much as the starter (ST) reference strain *S. cerevisiae* (Table 4, Supplementary Figure S3 and Table S2). The lowest concentration of isopentyl alcohol was detected in wine produced by the H1 strain. However, this amount was very similar to the starter strain. Isobutanol is a precursor of fruit esters and its concentration in wine fermented by H2 hybrid strain was almost three times higher than in the starter-fermented, wine samples and significantly lower than the amount in the *S. kudriavzevii*-fermented wine. Alcohol level of the wine produced with the H1 strain was similar to that of the starter strain. This was not however the case for glycerol content. Increased glycerol content gives wines a sweet taste. In line with its positive effect, the glycerol production capacity of the starter strain has recently become an important parameter in the selection process of autochthonous yeasts. The lowest glycerol content was produced by strain H1, whereas the highest glycerol content was detected in wines produced with the reference strain *S. kudriavzevii* (Table 4, Supplementary Figure S3 and Table S2).

Nine esters were monitored as this chemical group is an important determinant of aromatic character. In this study, ethyl-acetate was the dominant ester in every wine, ranging from 10.86 mg/L to 31.06 mg/L. The level was 75–92% of the total ester content in the wine samples; the other components were detected at very low levels. At low concentrations (50 mg/L or below), ethyl-acetate contributes positively to the overall complex bouquet. The wine made by the H2 hybrid yeast contained the highest amount of ethyl-acetate, (pineapple flavour), hexyl-acetate (apple, plum flavour) and isopentyl-

acetate (pear, banana flavour). These compounds belong to the group of fruit esters mentioned earlier. They provide a variety of fruity aromas and flavours that are popular with consumers and are mainly formed at low fermentation temperatures (10–20 °C). Wine samples fermented with *S. paradoxus*, starter yeast and the H2 hybrid strain had higher levels of total esters. The hybrid yeast produced higher or equal amounts of all esters tested compared to the potential representative parental species (*S. kudriavzevii* and *S. cerevisiae* starter) (Table 4, Supplementary Figure S3 and Table S2).

The terpenes are mainly characteristic of muscatel grape varieties, their presence may influence the complex flavour and aroma. Four terpenes were investigated in the wine samples, but the concentrations of α-terpineol and geraniol were both below the detection limit. The linalool level in the samples was very low, between 0.0–0.01 mg/L, which did not allow its use in the analysis and the amount of β-citronellol was the same in all samples.

Principal component analysis (PCA) was used to visualize the effects of different yeast strains on the volatile compounds using "Kéknyelű" grape juice in laboratory scale fermentation (Supplementary Figure S4). The PCA clusters were mainly based on the PC1 trait (Supplementary Table S3). Butanoic acid, α-terpineol and geraniol were not detected in the samples, while the 1-hexanol, linalool and β-citronellol did not show significant differences within the samples. However, the other examined compounds showed differences within the samples with high statistical significance. As it was expected, the samples clustered into six groups corresponding to the investigated yeast strains during the PCA analysis (Supplementary Figure S4). It provided evidence that the yeast selection can highly influence or even determine the characteristic component patterns of the wine produced from same grape juice.

4. Discussion

In this study we identified two isolates from grape juice using PCR based techniques and investigated their oenological potential and their ability to produce important volatile compounds that affect wine quality. We reported important information on the strains that influence the efficiency of the fermentation process and play an important role in organoleptic characteristics of wine.

According to species-specific PCR results, the investigated primer pairs showed good specificity, but we could not prove the hybrid nature of the H1 and H2 strains, so we performed PCR-RFLP analyses based on *CYR1* and *HIS4* genes. The results indicated that the H1 isolate is not clearly identifiable, as the RFLP pattern does not allow a clear distinction between *S. cerevisiae* and *S. paradoxus* species. Indeed, according to Muir and colleagues [35] these primers are not reliable for the identification of some isolates, including hybrid strains. Thus, H1 could belong to *S. cerevisiae* or *S. paradoxus* species, and it is also possible that it could be characterized as a hybrid of these two species. The H2 isolate can be treated as a hybrid of *S. cerevisiae* or *S. paradoxus* and *S. kudriavzevii* species. Summarizing the results of the RFLP analysis, we can conclude that H1 is most likely *S. cerevisiae*, while H2 is a hybrid of *S. cerevisiae* and *S. kudriavzevii*.

In this work we have compared the oenological character of two isolates to a representative *S. cerevisiae* (a commercial strain) and the type strains *of S. kudriavzevii*, *S. pastorianus* and *S paradoxus*. The putative parental type strains were chosen for the physiological test and the fermentation test, based on molecular identification methods.

Temperature affects the growth and metabolism of yeast strains and influences the composition of wine due to the secondary metabolism of yeasts. The result of the temperature tolerance test demonstrated that the isolates, H1 and H2 grow better at lower temperature (16 °C) compared to the *Saccharomyces* reference strains. A similar observation was made for *S. cerevisiae* × *S. kudriavzevii* hybrids [7,8,45]. This may be beneficial for ester production during the fermentation process and increase the fruity aroma of the wine [8]. In this test, the H2 hybrid isolate tolerated high temperature (37 °C), whereas the *S. kudriavzevii* putative parental species was not able to grow. The H2 strain showed more intensive growth at 16 °C compared to the control *S. cerevisiae* strain. These results indicated

that the H2 isolate combines the temperature tolerance of both putative parental species, which may indicate the hybrid nature of the isolate. Our results are in agreement with data reported in the literature [7,8,45], which show that *S. cerevisiae* strains are less capable to growth at low temperature in contrast to most *S. cerevisiae* × *S. kudriavzevii* hybrids [46]. Both isolated strains (H1 and H2) have potential for white wine and red wine fermentation, according to their temperature tolerance. White wines are generally produced at lower fermentation temperatures (15–20 °C), while red wines are usually fermented at higher temperatures [47].

Both H1 and H2 isolates show good ethanol and glucose tolerance and similar glucose fermentation capacity as the reference strains. The *S. cerevisiae* × *S. kudriavzevii* hybrid has beneficial properties in terms of wine making, for instance the high glucose and ethanol tolerance which are characteristic of the *S. cerevisiae* parent. In addition, the fermentation ability at lower temperature and higher glycerol production are properties of *S. kudriavzevii* parental strain [7,48].

Sulphite tolerance is a desired trait for yeast strains used in wine making [49]. It was reported that in a spontaneous wine fermentation, 50 mg/L sulphur dioxide (SO_2) in general is sufficient to inhibit most of the non-*Saccharomyces* yeasts found in grape juice, except *Candida* spp., while the addition of 20 mg/L inhibited only some of the non-*Saccharomyces* yeasts [50]. Strains of *S. cerevisiae* have been shown to be fairly tolerant to sulphite in general when compared with other yeasts but display highly diverse SO_2 tolerances [51]. The observed high sulphur dioxide tolerance of the H1 and H2 isolates may make them suitable for winemaking applications.

Wine produced with the H2 hybrid yeast contained significantly less residual sugar (4.2 ± 1.88 g/L) compared to the wine made by H1 strain (45.1 ± 1.01 g/L). This demonstrates that the H2 strain can ferment more efficiently than the H1 isolate at low temperature. High residual sugar content and reduced fermentative capacity suggest that the H1 isolate cannot complete the fermentation after 20 days at 16 °C. However, it was characterised by the same glucose fermentation capacity and temperature tolerance at 16 °C as the H2 isolate. It was also found that the concentration of volatile acid was not significantly different between the strains studied, except *S. pastorianus*-H2, *S. paradoxus*-H2 and *S. pastorianus*-H1 (Supplementary Table S1).

In the fermentation experiment, the weight loss of the must inoculated with the isolated strains investigated and the reference strains was monitored. In this respect, the two most effective strains were the *S. cerevisiae* × *S. kudriavzevii* H2 hybrid and the *S. paradoxus* reference strain, while the reference strain *S. kudriavzevii* can be characterized by reduced fermentation capacity. In conclusion, the H2 hybrid strain exceeded the fermentation capacity of the putative parental species. The *S. cerevisiae* × *S. kudriavzevii* hybrid strain can ferment well at lower temperature (16 °C), which is in correlation the cold tolerance of H2 strain and the results of the fermentation capacity test at 16 °C.

In general, the aroma components are also highly dependent on the grape variety [52,53]. Since we have used a single grape variety ("Kéknyelű"), our conclusions are based primarily on data for this grape variety.

Different yeast strains can mobilize aroma compounds or aroma precursors from must into wine [54,55]. Also, higher alcohol contents and activity of alcohol acetyltransferases play a critical role in regulating the formation of acetate esters in wine. The amount of esters could depend on the concentration of acetyl-CoA because activated acetyl-CoA is a substrate of acetate ester biosynthesis [56]. The wine fermented with strain H2 contained higher levels of higher alcohols and this strain is capable of producing high concentrations of esters, especially ethyl-acetate. The majority of the esters have floral (rose, jasmine) and fruity (green apples, strawberries, pineapples) flavour, which contribute to the pleasant fruity aroma of the wines. Regarding the volatile compounds data, publications emphasize that it is not possible to infer the oenological properties of a wine from individual volatile compounds [7,8,11,14,45]. However, all studies generally agree that the composition of individual volatile compounds changes with respect to the strain used, usually due to the

presence of a higher amount of certain aromatic compounds (mainly esters). However, exactly which component changes cannot be consistently determined, as it may differ from hybrid to hybrid [7,8,11,14,45].

Our results support these observations, as four ester components (ethyl acetate, ethyl hexanoate, hexyl acetate, isopentyl acetate) in the wine made by the H2 hybrid strain showed the highest values among the tested strains. In these wines, we observed eight esters compounds with higher value compared to the potential parental *S. cerevisiae* and *S. kudriavzevii* species. Increased ester production of the hybrid strain was detected for almost all ester compounds investigated (in the case of eight esters from nine ester components). No consistently higher value was observed for the strain H1 compared to *S. cerevisiae*.

From the point of view of practical use, the H2 strain meets the requirements of modern oenological operations. Its low temperature tolerance is essential in reductive winemaking. The formation of esters is promoted under these conditions, and thus the development of fruity flavours and aromas and character. Ethanol and glucose tolerance, as well as glucose fermentability ensures the possibility of producing dry wines with higher alcohol content. This is an indication that it can also be used successfully as a starter culture in fermentation for other, high sugar containing grape varieties. The composition and quantity of aromas that emerge ensure the development of a pleasant fruity character.

Unfortunately, the H1 isolate does not meet the criteria of modern winemaking because of the incomplete fermentation. However, its killer activity is noteworthy, so it would be interesting to test its fermentation ability at higher temperatures in the future. Although *S. cerevisiae* is the most frequently occurring species in wine fermentation, interspecific hybrids are receiving more and more interest with respect to their potential to be used as starter cultures. The value of hybrids as starter cultures is based primarily on their ability to combine the beneficial traits of the two (or more) different parent species. These hybrids can be either produced under laboratory conditions or isolated from nature, and anthropogenic environments such as wineries. In this work, we have tested two yeast isolates and demonstrated that they have a number of advantageous traits. In this respect, one of the isolates, a hybrid (*S. cerevisiae* × *S. kudriavzevii*) has beneficial properties in terms of heat, ethanol, sulphur and glucose tolerance as well as the production of various volatile compounds, suggesting that it has good potential for use in winemaking.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8080416/s1, Table S1: Results of statistical analysis using ANOVA variance analysis, represents the *p*-value of the samples in case of residual sugar content, volatile acid concentration and pH; Table S2: Significant components identified by One-way ANOVA and post-hoc analysis; Table S3: PC1 and PC2 loading values of the volatile components PCA; Figure S1: Results of the physiological characterization I: glucose tolerance test in presence 30% glucose, ethanol tolerance using 30% ethanol, killer activity test, glucose fermentation test at 16 °C, temperature tolerance test at 5 °C, 16 °C, 24 °C and 37 °C; Figure S2: Results of the physiological characterization II: sulphur dioxide (SO_2) tolerance determined by microdilution methods; Figure S3. Differences in volatile compounds and glycerol in wines made with different starter yeasts; Figure S4 Principal component analysis (PCA) of the volatile components in the "Kéknyelű" wine inoculated with different yeast strains.

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