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Novel *Saccharomyces cerevisiae* × *Saccharomyces mikatae* Hybrids for Non-alcoholic Beer Production

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Abstract: The popularity of non-alcoholic beers has been increasing over the past few years. Maltose-negative strains of different genera are frequently used to obtain beers of low alcohol content. *S. cerevisiae* hybrids with other *Saccharomyces* species offer interesting inherited flavour characteristics; however, their use in non-alcoholic beer production is rare. In this work, we constructed six hybrids of maltose-negative *S. cerevisiae* parental strains (modified to produce higher amounts of organic acids) and *S. mikatae* (wild-type). Growth behaviour, osmotolerance and fermentation features of the offspring were compared with parental strains. One hybrid with mitochondrial DNA inherited from both parents was used to produce non-alcoholic beer in which organic metabolites were evaluated by HPLC and HS-SPME-GC-MS. This hybrid produced non-alcoholic beer (≤0.05% (*v*/*v*)) with an increased organic acid content, just as its parent *S. cerevisiae*, but without producing increased amounts of acetic acid. The beer had a neutral aromatic profile with no negative off-flavours, similar to the beer produced by the parent *S. mikatae*, which was used for the first time to produce non-alcoholic beer. Overall, both parents and hybrid yeast produced non-alcoholic beers with increased amounts of higher alcohols compared with esters.

Keywords: non-alcoholic beer; *Saccharomyces mikatae*; *Saccharomyces cerevisiae*; hybrid yeast

Citation: Vaštík, P.; Sulo, P.; Rosenbergová, Z.; Klempová, T.; Dostálek, P.; Šmogrovičová, D. Novel *Saccharomyces cerevisiae* × *Saccharomyces mikatae* Hybrids for Non-alcoholic Beer Production. *Fermentation* **2023**, *9*, 221. https://doi.org/10.3390/fermentation9030221

Academic Editors: Frank Vriesekoop, Teodora Emilia Coldea and Elena Mudura

Received: 31 January 2023
Revised: 22 February 2023
Accepted: 22 February 2023
Published: 25 February 2023

1. Introduction

In recent years, increasing awareness about healthy nutrition and well-being has significantly affected trends in the food and beverage industry. The negative effect of alcohol on human health, together with regulations and religious beliefs, are shifting consumer's preferences toward non-alcoholic beverages [1,2]. Among alcoholic beverages, beer holds a prominent share of the global market, and even though non-alcoholic beers are becoming increasingly popular, they still account for only a minor proportion of total beer products [1]. The term "non-alcoholic" is associated with beers containing no more than 0.5% (*v*/*v*) ethanol [3], although worldwide, the limits may differ and be as low as 0.05% (*v*/*v*) [4]. Non-alcoholic beers are traditionally produced either by arresting the fermentation process or by removing ethanol from the fermented beer [5,6]. However, both of these methods have detrimental effects on the quality of the non-alcoholic beer; off-flavours and lack of aroma and complexity are only a few of the reasons why customers refrain from switching to non-alcoholic beers [4].

One of the strategies that has been extensively explored over the past decades uses maltose-negative yeast strains in beer fermentation [7]. Their inability to ferment maltose results in limited ethanol production, while the metabolism of simple sugars is sufficient for

the production of organic molecules that contribute to the beer's aromatic profile [8]. However, the products are often associated with "worty" or "cooked" flavours and undesirable turbidity and viscosity [9]. The use of naturally fruit-residing yeast strains with impaired maltose transport can significantly improve the aromatic profile of non-alcoholic beers, as they produce increased amounts of flavour-active compounds to attract insects [10]. Today, research focuses mainly on the use of maltose-negative non-*Saccharomyces* yeasts that produce notable amounts of flavour-active compounds, flocculate easily and are consumer-safe [2].

The enormous advancement in molecular biology has opened the door to the production of non-alcoholic beers with desired flavours and properties through the employment of genetically modified yeast in beer fermentation [11]. Even though the use of GMO is still frowned-upon by the general public, and has to be handled carefully, an increased number of studies has focused on the production of non-alcoholic beverages by genetically modified strains [12–19]. Members of the monophyletic clade of *Saccharomyces*, known as the *Saccharomyces sensu stricto* group, are able to undergo hybridisation, which can shape the hybrid to withstand harsh environments [20]. Moreover, alloploid strains that tend to be capable of producing viable diploid spores may be formed during hybridisation. Thus, allotetraploid inter-specific hybrids may undergo meiosis, enabling crossovers and gene conversions [21].

An excellent example of a natural inter-specific hybrid between *S. cerevisiae* and *S. eubayanus* is yeast known as *S. pastorianus* and is the powerhouse of the modern brewing industry [22]. Bellon et al. [23] described the first inter-specific hybrid formed between a *S. cerevisiae* wine strain and *S. mikatae*, which introduced more flavour complexity into the produced wine. Another hybrid between *S. mikatae* and *S. cerevisiae* showed increased production of compounds yielding fruity, banana and floral aromas in white wines [24]. However, there is limited information available about natural or synthetic hybrids between *S. mikatae* and *S. cerevisiae* [25,26].

This work provides a clearer insight into novel constructed yeast hybrids of maltose-negative *S. cerevisiae* parents (GMO) and *S. mikatae* (wild-type) targeted for non-alcoholic beer production. Maltose is the most abundant saccharide present in the beer wort [3], and here, we describe the application of *S. mikatae* for the first time in non-alcoholic beer production. Construction of hybrids and their use was led by the motivation to imitate the natural hybridisation event and reveal the influence of recombination on the inherited features of the offspring.

2. Materials and Methods

2.1. Media and Solutions

YPD: (10 g L^{-1} yeast extract (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA), 10 g L^{-1} peptone (Thermo Scientific™, USA), 20 g L^{-1} glucose (Merck, Darmstadt, Germany), pH 6.2). **MIN** (minimal medium): (0.17% (*w*/*v*) YNB (Yeast Nitrogen Base) (HiMedia®) without ammonium sulfate; 0.5% (*w*/*v*) ammonium sulfate (Lachema, CZ); 2% (*w*/*v*) glucose (Merck, Darmstadt, Germany)). **MING$^+$**: (MIN + 200 µg mL^{-1} of Geneticin™ (Thermo Scientific™, USA)], **YPDA**: [**YPD** + 20 g L^{-1} agar (Carl Roth, GmbH, Germany), pH 6.2). **YPDAG$^+$**: (**YPDA** + Geneticin™ (200 µg mL^{-1}) (Thermo Scientific™, USA), pH 6.2). For screening and regular maintenance, the strains were kept on agar plates (20 g L^{-1} agar (Carl Roth, GmbH, Germany)) with or without Geneticin™ (200 µg mL^{-1} (Thermo Scientific™, USA)). **Lysate solution B**: (0.05 mol L^{-1} Tris-HCl (Merck, Darmstadt, Germany) pH 7.5; 0.02 mol L^{-1} EDTA (VWR, USA) pH 7.5; 0.1% (*w*/*v*) SDS (Sigma Aldrich, St. Louis, MO, USA)), TBE solution: (108 g of Tris-HCl (Merck, Darmstadt, Germany), 55 g of boric acid (Lachema, CZ), 40 mL 0.5 mol L^{-1} EDTA (VWR, USA) pH 8.0). **Saccharide solutions**: (10 g L^{-1} yeast extract (Oxoid, ThermoFisher Scientific, USA), 20 g L^{-1} glucose or maltose/maltotriose/sucrose (Merck, Darmstadt, Germany), 0.001% (*v*/*v*) bromothymol blue (Merck, Darmstadt, Germany), and 1 mol L^{-1} K_2CO_3 (CentralChem, Bratislava)). **10°P**, **8°P** and **7°P worts**: (Pilsen malt (Vikings Pilsen Malt) and Žatecký poloraný červenák

hop pellets by infusion mashing in a 20 L microbrewery Braumeister (Speidel, Ofterdingen, Germany)). The mashing regime used was: 10 min at 38 °C, 30 min at 52 °C, 60 min at 65 °C, 60 min at 75 °C, 10 min at 78 °C. Boiling was held for 60 min at 100 °C, and hop pellets were added at 60 min, 45 min and 15 min of the boil. The wort was then cooled to 20 °C and used for further experiments.

2.2. Microorganisms

Hybrid yeasts were constructed at the Faculty of Natural Sciences of the Comenius University in Bratislava, Slovakia. Modified deletion mutants of *Saccharomyces cerevisiae* Δ*ACO1 G418*^{R}, *Saccharomyces cerevisiae* Δ*KGD1 G418*^{R} and *Saccharomyces cerevisiae* Δ*CIT1 G418*^{R} were constructed by the replacement of wild-type genes with the kanMX4 allele [27] and were obtained from a systematic deletion project [28]. Parental *Saccharomyces mikatae* CBS 8839T was obtained from the Central Bureau of Fungal Cultures in the Netherlands. Commercial brewer's strains *Saccharomyces cerevisiae* K97 and *Saccharomyces pastorianus* W34/70 were obtained from a local supplier. All yeasts used in this work are listed in Table 1 and primers are listed in Table 2.

Table 1. List of hybrids, their parents and brewer´s controls used in this work.

Yeast	Genotype	Abbreviation (Type)
Saccharomyces mikatae CBS* 8839T	Wild type	*Sm* (parent)
Saccharomyces cerevisiae Δ*ACO1 G418*^{R}	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, aco1::kanMX4	*ACO1* (parent)
Saccharomyces cerevisiae Δ*KGD1 G418*^{R}	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, kgd11::kanMX4	*KGD1* (parent)
Saccharomyces cerevisiae Δ*CIT1 G418*^{R}	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, cit1::kanMX4	*CIT1* (parent)
S. mikatae CBS 8839T × *S. cerevisiae* Δ*ACO1 G418*^{R}		*Hyb1ACO1* (hybrid)
S. mikatae CBS 8839T × *S. cerevisiae* Δ*ACO1 G418*^{R}		*Hyb2ACO1* (hybrid)
S. mikatae CBS 8839T × *S. cerevisiae* Δ*KGD1 G418*^{R}		*Hyb1KGD1* (hybrid)
S. mikatae CBS 8839T × *S. cerevisiae* Δ*KGD1 G418*^{R}		*Hyb2KGD1* (hybrid)
S. mikatae CBS 8839T × *S. cerevisiae* Δ*CIT1 G418*^{R}		*Hyb1CIT1* (hybrid)
S. mikatae CBS 8839T × *S. cerevisiae* Δ*CIT1 G418*^{R}		*Hyb2CIT1* (hybrid)
Saccharomyces cerevisiae K97		K97 (top fermenting control)
Saccharomyces pastorianus W34/70		W34/70 (bottom fermenting control)

* CBS = Central Bureau of Fungal Cultures (The Netherlands), G418^R = resistant to geneticine

Table 2. Primers used in this work [29].

Direction	Name of Primer	Sequence 5′→3′
Forward	D1/D2-NL1	GCATATCAATAAGCGGAGGAAAAG
Reverse	D1/D2-NL4	GGTCCGTGTTTCAAGACGG

2.3. The Construction and Verification of Hybrids

Prior to breeding, yeast cells were cultivated at 28 °C on YPDA medium. Ascospores were exposed to 50 µL of 0.5 mg mL^{-1} Zymolyase 20T (Amsbio, USA), incubated at 30 °C for 15–30 min and vortexed. After ascus disintegration, exposed spores were mixed with an equivalent number of cells of the opposite yeast strain and poured onto YPDA. Hybridisation or formation of zygotes was controlled microscopically. Yeasts were maintained on YPDA and YPDAG$^+$ medium at 4 °C. All yeasts (Table 1) were inoculated onto a selective MING$^+$ medium, on which only inter-species hybrids were able to grow. The parental prototrophic strain *S. mikatae* was G^S (Geneticin sensitive), and parents of *S. cerevisiae* were G^R (Geneticin resistant) and contained auxotrophic mutations (leu-, his-, ura-). Isolation of gDNA (genomic DNA) was performed in accordance with [30], and the mtDNA (mitochondrial DNA) was purified by differential centrifugation methods described in [31].

Amplification of DNA was performed in a Mastercycler thermocycler (Eppendorf, Hauppauge, NY, USA). Template DNA was amplified in 25 µL of reaction solution: 12.3 µL of miliQ; 2.5 µL 10× B PCR buffer; 2.5 µL 2 mmol L^{-1} of dNTP; 2.5 µL of 25 mmol L^{-1} $MgCl_2$; 1 µL 25 pmol. μL^{-1} of forward primer; 1 µL 25 pmol. μL^{-1} of reverse primer; 0.2 µL Taq DNA polymerase (5 U) (FIREPOL®); 0.5 µL gDNA. The amplification quality was checked on a 1% (*w/v*) agarose gel by electrophoresis. Program: 94 °C (3 min), 35 × (94 °C—45 s, 55 °C—2 min, 72 °C—2 min), 72 °C—5 min, 14 °C. Confirmation of hybridisation was performed by PCR *Hae*III polymorphism of the D1/D2 domain of the 26S rRNA gene in accordance with [32]. Isolated DNA from each yeast colony was amplified with the NL1 and NL4 primers (Table 2), PCR products were digested with *Hae*III enzyme and analysed by electrophoresis on an agarose gel. mtDNA evaluation was performed according to [33] as follows: gDNA was cleaved by *Hinf*I (Takara Bio, USA)estriction en rzyme (5′ G↓ANTC 3′; 3′ CTNA↑G 5′). The content of a 40 µL of *Hinf*I reaction mixture was: 19 µL of gDNA; 15 µL miliQ; 4 µL 10× buffer; 1 µL of RNase A (Serva, Germany) (10 mg.mL^{-1}); 1 µL *Hinf*I. A total of 2 drops of paraffin oil were added to the mixture, and the solution was cultivated overnight at 37 °C. The restriction mixture was extracted with a mixture of phenol–chloroform and centrifuged (12,000× *g*, 15 min, 4 °C). A total of 20 µL of restriction mixture was added onto a 1% (*w/v*) agarose gel. The DNA was separated in 1% (*w/v*) agarose gel in TBE solution, which contained 0.5 µg mL^{-1} of ethidium bromide (Sigma Aldrich, USA). DNA was visualised under the UV light at 312 nm, and the lengths of DNA fragments were compared with a standard λ/*Pst*I 300 ng ladder.

2.3.1. Yeast Starters

Yeast starters used in experiments were prepared in a 24 h submersed cultivation of individual yeast strains in 20 mL of liquid YPD medium in 100 mL Erlenmeyer flasks on an orbital shaker (Biosan ES-20, Riga, Latvia) at 2 Hz, 28 °C. Cell counting for each yeast strain was performed using a Leica DM 2500 light microscope (Leica Microsystems) with a Bürker chamber.

2.3.2. Sugar Fermentation

The ability of yeast strains to ferment various sugars (glucose, maltose, maltotriose, sucrose) was tested in glass tubes containing inverted Durham tubes. A total of 5 mL of saccharide solution with bromothymol blue as an acid/base indicator and 1 mol L^{-1} K_2CO_3 as a buffering agent were inoculated with 10^6 cells mL^{-1} of liquid yeast starter. Production of CO_2, indicating saccharide fermentation, was evaluated in Durham tubes following static cultivation at 25 °C for 10 days. The cultivations were performed in triplicate.

2.3.3. Osmotolerance

To determine sensitivity of strains to different conditions, 10^6 cells mL^{-1} of liquid yeast starter were cultivated at 20 °C for one week in sterile glass tubes, each containing 10 g L^{-1} of yeast extract and different concentrations of glucose (0; 1; 2; 5; 10; 30 and 50) in % (*w/w*). Osmotolerance was determined by A_{600nm} measurements, where each non-inoculated solution of a specific concentration was used as a blank against a medium of a specific concentration with inoculum. Experiments were performed in triplicate.

2.3.4. Aerobic Growth

Growth of yeast strains was evaluated using YPD medium. The cultivations were performed in 96-well microtiter plates (Sarstedt, Germany) filled with 200 µL of medium and inoculated with 10^6 cells mL^{-1} of liquid yeast starter. Plates were incubated at 25 °C and shaken (4 Hz) in a Varioskan® Flash microplate reader (ThermoFisher Scientific, Waltham, MA, USA). Growth was monitored by measuring the absorbance at 600 nm (OD_{600nm}) every hour for 2 days.

2.3.5. Phenotype verification of *Saccharomyces cerevisiae* Mutants

For phenotype verification of *Saccharomyces cerevisiae* mutant yeast (*ACO1*, *KGD1* and *CIT1*), 8°P and 10°P worts were used. A total of 48 mL of wort in 50 mL fermentation flasks were inoculated with yeast at a cell concentration of 10^6 cells mL^{-1}. Flasks were sealed, and fermentation was performed at 12 °C for 7 days. Young beer samples were analysed for organic acid concentrations by HPLC.

2.4. Beer Production

For beer production, 480 mL of 7°P wort in 500 mL fermentation PET flasks were inoculated with yeast starters to achieve a starting biomass concentration of 10^6 cells mL^{-1}. Flasks were closed and fermentation proceeded at 18 °C for one day and then at 3 °C for a month. Beer samples were analysed by HPLC (sugars, organic acids, ethanol and glycerol) and by HS-SPME-GC-MS analysis for volatile organic compounds (VOCs) (esters, higher alcohols). Each beer fermentation was performed in triplicate.

2.5. Analytical Methods

Ethanol concentration, original extract, real degree of fermentation, pH and colour of beer samples was determined using a density meter DMA 4500M coupled with an Alcolyzer Beer ME, Haze QC ME Turbidity Measuring Module and pH ME Beverage Measuring Module (Anton Paar, GmbH, Graz, Austria).

2.5.1. Analysis of Beer by HPLC-RID

Before analysis, the beer samples were centrifuged (10 min, 2511× *g*) and supernatants were diluted with deionised water. An Agilent 1260 HPLC system (Santa Clara, CA, USA) coupled to an RI (refractive index) detector using an Aminex HPX-87H column (300 mm, 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) was used. Sulfuric acid (5 mmol L^{-1}) was used as the mobile phase, with a flowrate of 0.6 mL min^{-1}. Separation was performed at 25 °C, and the injection volume was 20 µL. Signal detection was carried out using a refractive index detector (RID). Accurate concentrations of glucose, maltose, maltotriose, ethanol and glycero, and acetic, citric, malic and succinic acid were determined using the single standard addition method. Standards with purity ≥ 99% were obtained from Merck (Darmstadt, Germany). Each beer sample was analysed in triplicate.

2.5.2. Analysis of Beer by HS-SPME-GC-MS

Prior to analysis, beer samples were cooled and stored at 4 °C. A total of 50 mL of each beer sample were centrifuged (10 °C, 5054× *g*, 10 min), and the supernatant was poured into a 50 mL flask and closed. Flasks were shaken for 3 min to remove the CO_2. In the meantime, 2 g of NaCl with (≥99.9% purity, Pentachemicals, Czech Republic) were put into 20 mL darkened vials together with 10 mL of beer sample and 100 µL of internal standard (IS) solution, which contained ethyl heptanoate (≥ 99% purity, Sigma Aldrich, DE) and 3-octanol (≥ 99% purity, Sigma Aldrich, USA). Each vial was vortexed for 30 s to dissolve the NaCl and homogenise the sample. Headspace solid-phase microextraction (HS-SPME) of volatile organic compounds (VOCs) was performed for 30 min at 50 °C using an 85 µm Carboxen/polydimethylsiloxane (CAR/PDMS) fibre (Supelco, USA). VOCs were separated and determined using an Agilent GC 6890N system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5975B single quadrupole mass spectrometer detector (MSD) (Agilent Technologies, USA) and to a headspace solid-phase microextraction (HS-SPME) autosampler (COMBI PAL CTC Analytics, Switzerland). Analytes were separated in a DB-624 capillary column (30 m × 0.25 mm × 1.40 µm) (Agilent Technologies, USA). VOCs were desorbed in the injector of the GC in splitless mode for 10 min, and the temperature was set at 260 °C. Helium gas was used at a flow rate of 1 mL min^{-1}. The initial temperature program of the oven was set at 30 °C and held for 10 min, followed by three ramps in which the gradient was 2 °C/min until reaching 52 °C and held for 2 min, then 2 °C/min to 65 °C and held for 2 min, and lastly, at 5 °C/min to 250 °C and held for 3 min. The ionisation

energy was 70 eV, and detection and data acquisition were performed in a scan mode from 20 to 500 Da.

Identification of compounds obtained in the GC-MS analysis were compared with *m/z* values collected in the NIST MS spectrum library, Search version 2.0 (National Institute of Standards and Technology, Gaithersburg, MD, USA). Verification of compound identity was carried out by comparison of their MS spectra and retention times with standards. Quantification was performed using an IS (internal standard) and standard calibration curves for ethyl acetate (purity ≥ 99.7%), 2-methyl-1-propanol (≥99.5%), propyl acetate (≥98%), 3-methyl-1-buthanol (≥98.5%), 2-methyl-1-butanol (≥98%), 2-methylpropyl ethanoate (isobutyl acetate) (≥99%), ethyl butanoate (≥95%), butyl acetate (≥100%), butane-2,3-diol (≥98%), 3-methylbutyl acetate (isoamyl acetate) (≥98%), furfuryl alcohol (≥98%), ethyl hexanoate (≥99%), hexanoic acid (≥99.5%), linalool (≥95%), 2-phenylethanol (≥99%), ethyl octanoate (≥98%), octanoic acid (≥98%), 2-phenylethyl acetate (≥99%), 4-vinylguaiacol (≥98%), ethyl decanoate (≥99%) and decanoic acid (≥98%). Standards were obtained from Sigma Aldrich, USA. Calibration was carried out at three different concentrations and each point was performed in triplicate. VOCs of beer samples were measured in triplicate, and average values were taken.

2.6. Statistical Methods

All fermentation experiments in this study were performed in triplicate. The statistical analysis of the chromatographic VOC profiles and the principal component analysis (PCA) were performed using Statistica 12 software (StatSoft, USA). The PCA was used to explain the differences between beers, and results were explained by visualisation of the first two principal components on the scatter plot.

3. Results and Discussion

3.1. Phenotype Verification of Saccharomyces cerevisiae Mutants

The purpose of this work was to evaluate the potential of *S. cerevisiae* hybrids to produce non-alcoholic beers. In order to verify the phenotype of deletion mutants of *S. cerevisiae* (*ACO1*, *KGD1* and *CIT1* [15]), trial fermentations with 8°P and 10°P worts were performed. As presumed, mutant strains produced increased amounts of organic acid compared to standard brewer's strains *S. cerevisiae* K97 and *S. pastorianus* W34/70 from both 8°P and 10°P worts (Table 3). However, all mutant strains of *S. cerevisiae* also produced increased amounts of acetic acid, which drastically influenced the beer flavour through its sharp, sour and vinegar-like taste if present above the threshold level of 200 mg L^{-1} [34].

Table 3. Concentration of organic acids (g L^{-1}) in beers produced from 8°P and 10°P worts at 12 °C after 7 days.

	Citric Acid		Malic Acid		Succinic Acid		Acetic Acid	
Producer	**8°P**	**10°P**	**8°P**	**10°P**	**8°P**	**10°P**	**8°P**	**10°P**
S. cerevisiae ACO1	0.32 ± 0.01	0.45 ± 0.04	0.54 ± 0.01	0.62 ± 0.02	0.29 ± 0.05	0.31 ± 0.01	0.22 ± 0.03	0.66 ± 0.03
S. cerevisiae KGD1	0.22 ± 0.01	0.47 ± 0.04	0.53 ± 0.01	0.55 ± 0.02	0.32 ± 0.01	0.74 ± 0.02	0.23 ± 0.02	0.64 ± 0.03
S. cerevisiae CIT1	0.37 ± 0.01	0.52 ± 0.03	0.63 ± 0.05	0.73 ± 0.02	0.11 ± 0.00	0.31 ± 0.01	0.21 ± 0.02	0.62 ± 0.02
S. cerevisiae K97	0.07 ± 0.00	0.28 ± 0.01	0.04 ± 0.00	0.15 ± 0.00	n.d.	n.d.	n.d.	0.04 ± 0.00
S. pastorianus W34/70	0.08 ± 0.00	n.d.	0.04 ± 0.00	0.16 ± 0.00	n.d.	n.d.	n.d.	0.06 ± 0.00

Values are represented as: Average ± Standard Deviation, "*n*"—the number of replications of each beer fermentation was 3 and the number of each beer analysis was 3. ($n = 3 \times 3 = 9$), Note: n.d.—not detected

3.2. The Preparation of Yeast Hybrids

To confirm the correct hybridisation further, we analysed the *Hae*III restriction polymorphism of the D1/D2 domain of the 26S rRNA gene with PCR. From single yeast colonies, we isolated the DNA, amplified it using NL1 and NL4 primers (Table 2) and cleaved the PCR products with *Hae*III. DNA fragments were separated on an agarose gel. In the case of *Saccharomyces cerevisiae* mutant strains (*ACO1*, *KGD1* and *CIT1*), we anticipated band lengths of 133 bp, 160 bp and 322 bp. In the case of the *S. mikatae* genome,

band lengths of 160 bp and 457 bp were expected. All the hybrids had shown the mixed restriction profile (Figure 1) and were classified as true hybrids.

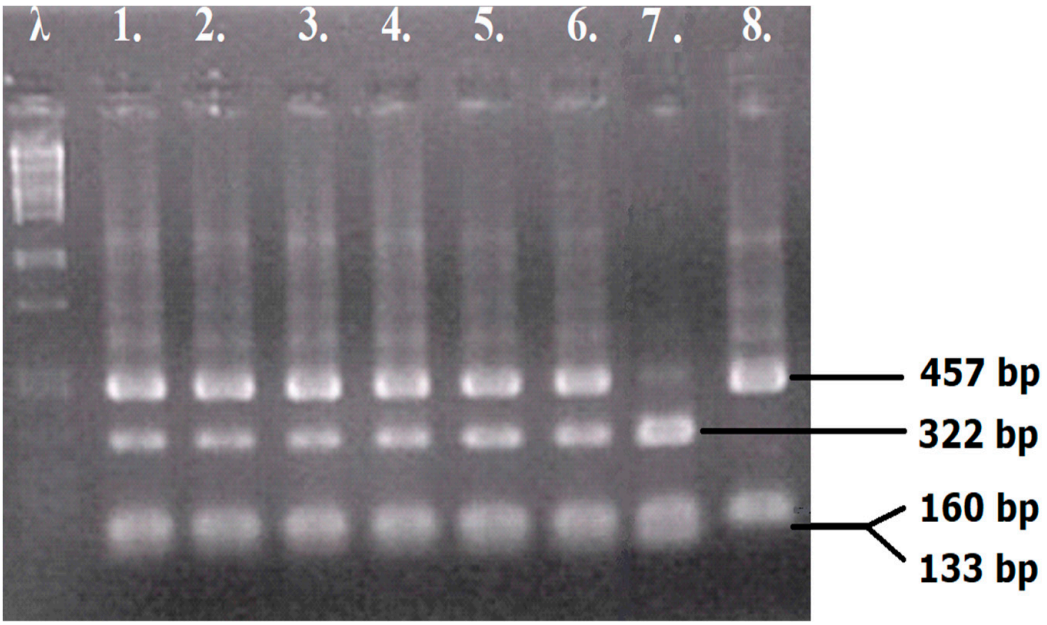


Figure 1. The analysis of 26S rRNA *Hae*III restriction polymorphisms of constructed hybrids (1(*Hyb1ACO1*), 2(*Hyb2ACO1*), 3(*Hyb1KGD1*), 4(*Hyb2KGD1*), 5(*Hyb1CIT1*), 6(*Hyb2CIT1*)) and parental strains 7(*S. cerevisiae, CIT1*) and 8(*S. mikatae, Sm*). λ(λ/*Pst*I) were used as the ladder.

Inter-species hybrids from the group of *Saccharomyces* are commonly present in the natural environment and are increasingly implemented in industrial productions. The best example of a naturally created hybrid, which is now commonly used in the production of lager beer, is *S. pastorianus* (*S. cerevisiae* × *S. eubayanus*). *S. pastorianus* inherited mitochondrial genes that are responsible for ethanol- and cryo-tolerant phenotypes [35,36]. The mitochondrial genome (mtDNA) of *S. cerevisiae* contains genes important for respiration, encoding proteins for cytochrome c oxidase (*cox1*, *cox2*, *cox3*), apocytochrome b (*cob*), three subunits of F_0 ATP synthase complex (*atp6*, *atp8*, *atp9*) and one ribosomal subunit rps3 [37]. Generally, in yeast, the mitochondria are inherited biparentally [38]. During the crossing of two haploids of *S. cerevisiae*, the mtDNA recombines, leading to heteroplasmy lasting for up to 20 generations. After that, cells achieve homoplasmy and retain either parental mitochondrial genomes or the recombined ones [39]. Mimicking natural hybridisation (by crossing mutant *S. cerevisiae* and *S. mikatae*) could generate offspring with beneficial features for the brewing industry (increased organic acid [15] and ester production [24]).

To reveal which forms of mtDNA the hybrids inherited, total genomic DNA was cleaved with *Hinf*I, and DNA fragments were separated on an agarose gel (Figure 2). Most of the hybrids inherited the mtDNA from parental *S. mikatae* (lines 1, 3, 4 and 6, Figure 2). However, one hybrid *Hyb2ACO1* (line 2, Figure 2) received the mtDNA from *S. cerevisiae*. Results also showed that hybrid *Hyb1CIT1* (line 5, Figure 2) possessed the recombinant mtDNA from both parental strains. Due to this interesting feature, the *Hyb1CIT1* hybrid was studied in terms of the production of non-alcoholic beer from a 7°P wort.

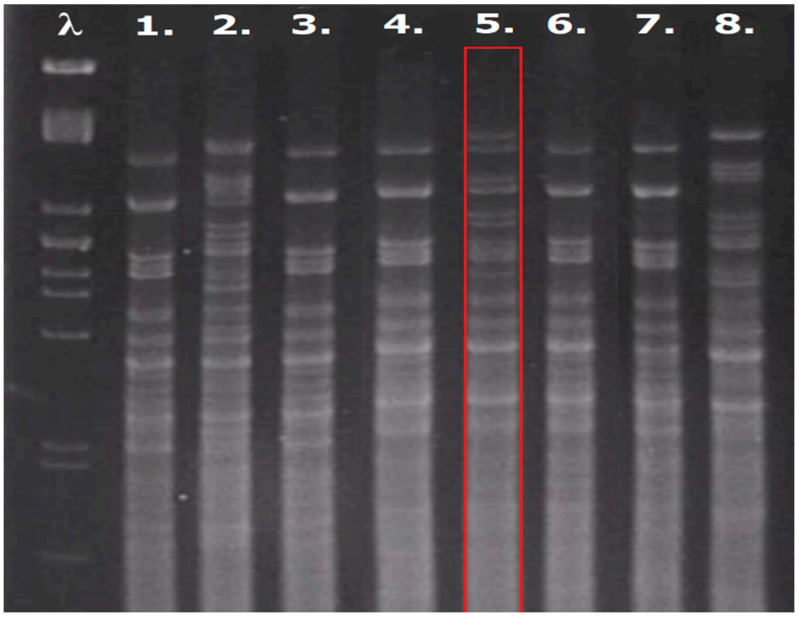


Figure 2. *Hinf*I restriction analysis of the total genomic DNA of hybrids. λ(*Pst*Iλ): ladder, 1(*Hyb1ACO1*), 2(*Hyb2ACO1*), 3(*Hyb1KGD1*), 4(*Hyb2KGD1*), 5(*Hyb1CIT1*), 6(*Hyb2CIT1*), 7(*S. mikatae*), 8(*S. cerevisiae CIT1*). Red section (line 5) represents hybrid yeast which inherited mtDNA from both parental strains.

3.3. Saccharide Fermentation Tests

As maltose contributes to the majority of fermentable sugars in wort [40], yeast with maltose or maltotriose-negative phenotypes are frequently used to produce non-alcoholic beer [2]. Only of few species of *Saccharomyces* do not ferment maltose; therefore, we chose maltose-negative strains of *S. cerevisiae* and *S. mikatae* as parental strains for the hybridisation. Three different parental *S. cerevisiae* strains with mutations/deletions in genes encoding enzymes involved in the Krebs cycle (ACO1, aconitase; KGD1, α-ketoglutarate dehydrogenase; and CIT1, citrate synthase) were used in this work. Screening of the hybrids on YPD and MIN agar plates containing 200 µg mL^{-1} Geneticin^{TM} confirmed the successful insertion of the kanMX4 allele, as all the constructed hybrids were able to grow in the presence of the antibiotic. The fermentative abilities of the constructed hybrids were tested (Table 4), and we showed that all the strains were able to ferment glucose and sucrose but were unable to ferment maltose and maltotriose, which emphasises their potential in the production of non-alcoholic beer.

Table 4. The fermentation of saccharides of the constructed yeasts hybrids and standard brewer's yeasts using Durham tubes at 25 °C for 7 days.

	Fermentation			
Yeast	**Glucose**	**Maltose**	**Maltotriose**	**Sucrose**
Hyb1ACO1	+	−	−	+
Hyb2ACO1	+	−	−	+
Hyb1KGD1	+	−	−	+
Hyb2KGD1	+	−	−	+
Hyb1CIT1	+	−	−	+
Hyb2CIT1	+	−	−	+
S. cerevisiae K97	+	+	+	+
S. pastorianus W34/70	+	+	+	+

Note: "+" = positive formation of CO_2, "−" = negative formation of CO_2

3.4. Osmotolerance

Another important parameter for beer production strains is osmotolerance. Overall, the yeasts (parental and hybrid strains) were able to handle up to 10% (*w*/*w*) of glucose in the medium (Figure 3), The cell density, however, rapidly decreased with increasing glucose concentration. It is known that hyperosmotic stress (caused by high concentrations of saccharides or ethanol) causes rapid shrinking of the yeast cells and activates the high-osmolarity glycerol response pathway [41]. Mutant *S. cerevisiae* parents as well as *S. mikatae* parents were able to withstand 30% (*w*/*w*) glucose, which is notably higher than the ability of their hybrid offspring.

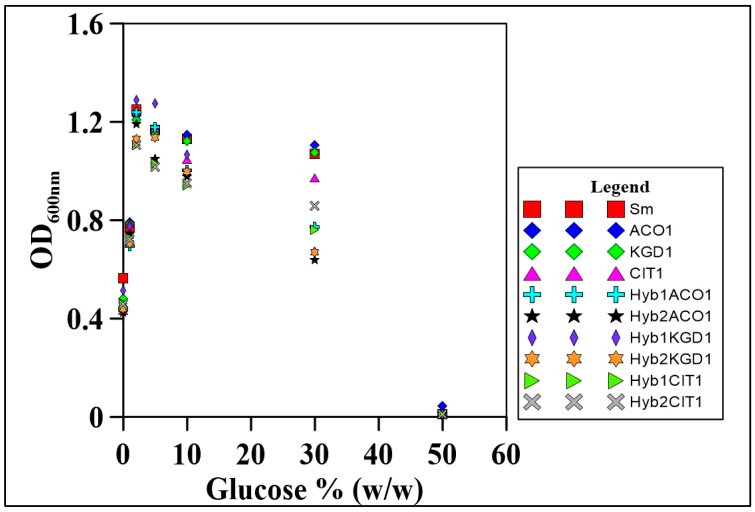


Figure 3. Osmotolerance of constructed hybrids and their parental strains *S. mikatae* (*Sm*) and *S. cerevisiae* (*ACO1*, *KGD1* and *CIT1*).

3.5. Aerobic Growth

No significant differences were detected among the hybrids in terms of aerobic growth (Figure 4). Overall, growth curves (Figure 4) of all hybrids were similar and displayed lower absorbances, representing lower growth rates than those of parental strains *S. mikatae* and *S. cerevisiae*. Hybridisation that brings alleles together in recombination may result in unpredictable results, with the outcome of reduced hybrid fertility, viability or even growth rate, but in some cases can also boost hybrid fitness [42].

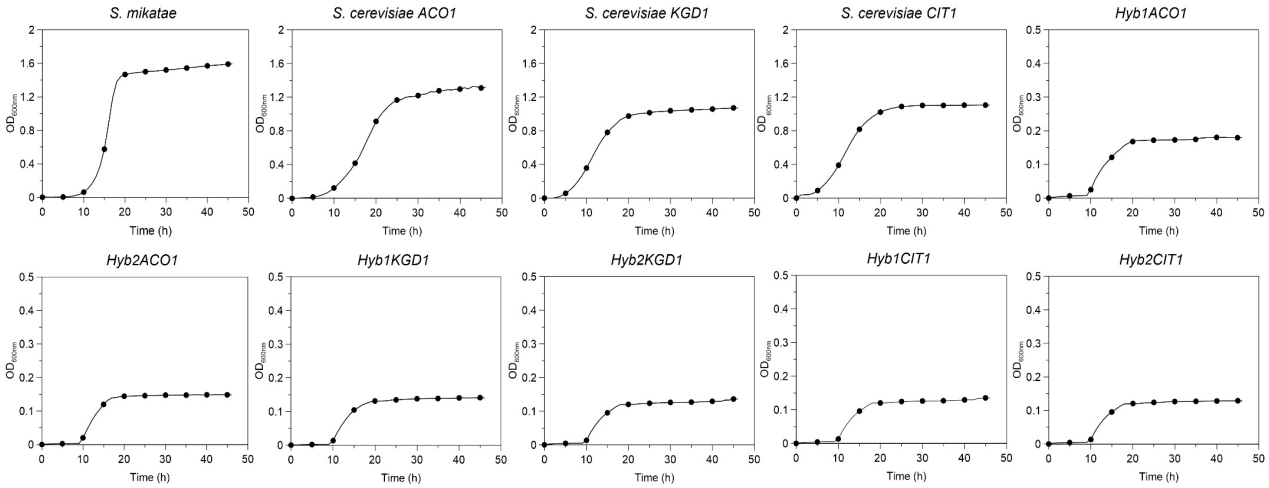


Figure 4. Aerobic growth of parental and hybrid strains in liquid 2% (w/w) YPD medium at 25 °C for 48 h. Each curve point represents an average value of 3 replications.

3.6. Beer Production

In order to investigate the fermentation performance of the production strains (Table 1) in a real setting, a 7°P wort was used for the production of beer using the parental and hybrid strains. We picked the hybrid *Hyb1CIT1* with recombinant mtDNA inherited from both parental strains (Figure 2) to test the influence of hybridisation on the production of non-alcoholic beer. Firstly, we prepared 7°P wort, which contains a lower number of fermentable sugars than 8°P and 10°P worts, in order to limit the final ethanol concentration. The results shown in Table 5 prove that we successfully obtained three non-alcoholic beers with ethanol concentrations lower than 0.05% (*v*/*v*), which was very promising.

Table 5. Basic parameters of final beers (*Sm*, *CIT1* and *Hyb1CIT1*) produced from 7°P wort in 500 mL.

Sample	Alcohol % (*v*/*v*)	Original Extract (°P)	Real Degree of Fermentation (%)	pH	Colour (EBC)
7°P wort	0.00 ± 0.00	7.19 ± 0.01	0.00 ± 0.00	6.05 ± 0.03	19.34 ± 0.35
Sm	0.03 ± 0.00	7.17 ± 0.01	0.68 ± 0.01	5.63 ± 0.02	16.69 ± 0.28
CIT1	0.03 ± 0.00	7.18 ± 0.01	0.67 ± 0.07	5.59 ± 0.02	18.36 ± 0.35
Hyb1CIT1	0.02 ± 0.00	7.17 ± 0.01	0.49 ± 0.04	5.72 ± 0.04	17.35 ± 0.27

Values are represented as: Average ± Standard Deviation, "*n*": the number of replications of each beer fermentation was 3 and the number of each beer analysis was 3. ($n = 3 \times 3 = 9$)

Glucose, acting as the sole fermentable sugar in the 7°P wort, was present at a concentration of 4.50 ± 0.07 g L^{-1} (Table 6) and served as the only potential carbon source for yeast metabolism. Glycerol formation is a wasteful process in terms of metabolism; however, its importance resides in osmoregulation of the cell and low-temperature tolerance, and it also plays a role as a sink for reducing equivalents [43]. The glycerol concentration in alcoholic beer is typically between 1–2 g L^{-1} [35], and its formation can be influenced by aeration, temperature, pH, sugar concentration and sulphite content [43]. In a study of non-alcoholic beer production using non-*Saccharomyces* yeasts [44], the concentration of glycerol in beers ranged from 0.4 to 1.0 g L^{-1}. In this study, glycerol concentration in three prepared non-alcoholic beers was no more than 0.65 g L^{-1} (Table 6).

Table 6. Organic compounds (g L^{-1}) in final beers (*Sm*, *CIT1* and *Hyb1CIT1*) produced from 7°P wort.

Organic Compound	7°P Wort	*Sm*	*CIT1*	*Hyb1CIT1*
Glucose	4.50 ± 0.07	n.d.	n.d.	n.d.
Maltose	34.65 ± 0.67	33.51 ± 0.98	34.34 ± 0.65	33.64 ± 0.76
Maltotriose	8.42 ± 0.14	8.37 ± 0.28	8.38 ± 0.24	8.04 ± 0.38
Glycerol	n.d.	0.62 ± 0.10	0.65 ± 0.07	0.63 ± 0.08
Ethanol	n.d.	0.23 ± 0.01	0.23 ± 0.01	0.16 ± 0.00
Acetic acid	n.d.	n.d.	0.21 ± 0.01	n.d.
Citric acid	n.d.	0.17 ± 0.01	0.24 ± 0.03	0.23 ± 0.01
Malic acid	n.d.	0.35 ± 0.03	0.52 ± 0.09	0.55 ± 0.02
Succinic acid	n.d.	n.d.	n.d.	0.04 ± 0.00

Values are represented as: Average ± Standard Deviation, "*n*": the number of replications of each beer fermentation was 3 and the number of each beer analysis was 3. ($n = 3 \times 3 = 9$) Note: "n.d." = not detected.

Formation of organic acids in beers is related to the metabolic pathways (glycolysis and the Krebs cycle) [45]. These compounds can impart beer body with fruitiness and sour taste and influence the beer foam and microbial stability [45,46]. A comparison of organic acids in beer samples (Table 6) revealed that concentrations did not differ dramatically, which also corresponds to pH values detected in the beers (Table 5). However, mutant *S. cerevisiae CIT1* produced acetic acid (Table 6), which is considered an off-flavour in beer [47] and was slightly above its threshold level of 200 mg L^{-1} [34]. It is important to note that hybrid *Hyb1CIT1* did not produce acetic acid, which eliminates the problem with negative off-flavours introduced to the final product by its parental strain.

3.7. Production of Volatile Organic Compounds (VOCs)

The esters present in beer are classified as acetate and medium-chain fatty acid ethyl esters. The key esters that implement a fruity aroma into the beverage are ethyl acetate, isoamyl acetate, isobutyl acetate, phenylethyl acetate, ethyl hexanoate and ethyl octanoate [48]. These compounds are formed during the beer fermentation process by yeast metabolism and diffuse from the yeast cell to the medium [49]. Concentrations of esters in beer can be affected by hydrostatic pressure, wort aeration and its composition, and lastly, by altering the fermentation conditions [50]. However, their concentration in non-alcoholic beer is limited due to the maltose-negative phenotype of the strain used [51]. Our results showed that ethyl acetate was the most abundant ester present in the product. Beer prepared with a hybrid yeast (*Hyb1CIT1*) contained 0.262 ± 0.039 mg L^{-1} of ethyl acetate (fruity and sweet aroma), which is similar to the concentration produced by the parental strains (Table 7).

Table 7. Content of volatile organic compounds (VOCs) in beers (*Sm*, *CIT1* and *Hyb1CIT1*) produced from 7°P wort.

Organic Compound (mg L^{-1})	*Sm*	*CIT1*	*Hyb1CIT1*
Esters			
Ethyl acetate	0.245 ± 0.079	0.369 ± 0.090	0.262 ± 0.039
Propyl acetate	n.d.	n.d.	n.d.
Butyl acetate	0.018 ± 0.005	0.022 ± 0.005	0.014 ± 0.006
Isoamyl acetate	0.003 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Isobutyl acetate	0.010 ± 0.006	0.012 ± 0.003	0.010 ± 0.005
2-Phenylethylacetate	0.004 ± 0.001	0.002 ± 0.000	0.003 ± 0.001
Ethyl butanoate	0.029 ± 0.005	0.025 ± 0.003	0.026 ± 0.004
Ethyl hexanoate	0.274 ± 0.020	0.212 ± 0.018	0.208 ± 0.012
Ethyl octanoate	0.004 ± 0.001	0.003 ± 0.000	0.006 ± 0.002
Ethyl decanoate	0.014 ± 0.002	0.007 ± 0.001	0.025 ± 0.011
Higher Alcohols			
3-Methyl-1-butanol	0.714 ± 0.247	0.746 ± 0.132	0.884 ± 0.122
2-Methyl-1-propanol	1.381 ± 0.403	1.531 ± 0.207	1.638 ± 0.247
2-Methyl-1-butanol	0.371 ± 0.069	0.457 ± 0.067	0.485 ± 0.059
2-Phenylethanol	0.533 ± 0.070	0.435 ± 0.054	0.469 ± 0.025
Butane-2,3-diol	n.d.	n.d.	n.d.
Furfuryl alcohol	0.072 ± 0.037	0.210 ± 0.073	0.145 ± 0.072
Terpene alcohols			
Linalool	0.097 ± 0.011	0.088 ± 0.006	0.091 ± 0.003
Phenols			
4-Vinylguaiacol	0.466 ± 0.060	0.258 ± 0.051	0.425 ± 0.079
Fatty acids			
Hexanoic acid (caproic)	0.202 ± 0.051	0.173 ± 0.045	0.130 ± 0.057
Octanoic acid (caprylic)	0.010 ± 0.002	0.011 ± 0.003	0.009 ± 0.002
Decanoic acid (capric)	0.001 ± 0.000	0.001 ± 0.000	0.004 ± 0.002

Values are represented as: Average ± Standard Deviation, "*n*": the number of replications of each beer fermentation was 3 and the number of each beer analysis was 3. ($n = 3 \times 3 = 9$.) Note: "n.d." = not detected.

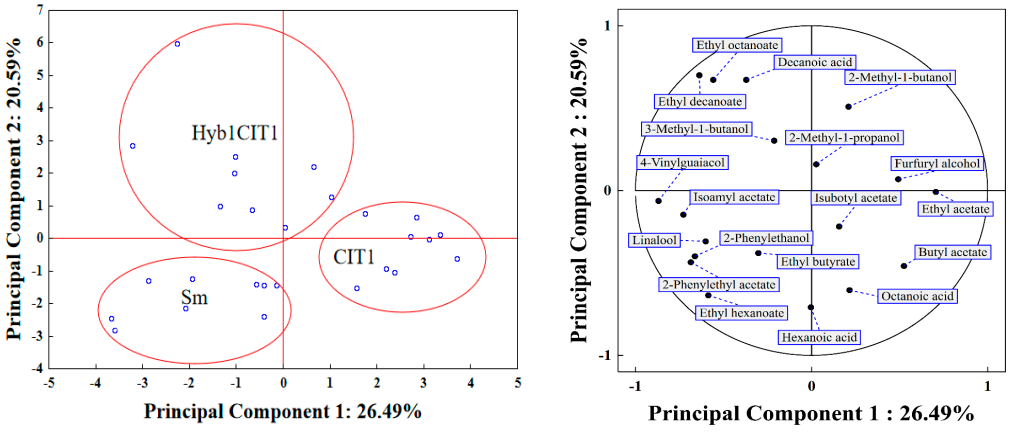
In the study by Catallo et al. [22], a hybrid cross between *S. cerevisiae* and *S. eubayanus* produced increased amounts of 3-methylbutyl acetate, ethyl acetate and ethyl hexanoate. The hybrids of *S. jurei* were also studied, and authors prepared beers with an interesting fruity profile [52], which supports the use of novel *Saccharomyces* hybrids in brewing. In this study, the banana flavour-inducing ester, iso-amyl acetate, with its threshold levels of 1.4–1.6 mg L^{-1} [4] and rose ester, 2-phenylethyl acetate, with a typical threshold level of 0.05–2.0 mg L^{-1} [53] were detected in beers in negligible concentrations (Table 7). From the so-called "apple" esters, ethyl hexanoate and ethyl octanoate [54], whose formation is directly connected to ethanol formation [49], ethyl hexanoate was detected in all three beers at similar concentrations (Table 7) but was slightly above the threshold level of 0.2 mg L^{-1} [55]. However, its sour apple aroma was not perceivable due to the sweet aroma caused by the residual maltose and maltotriose (Table 6).

Higher alcohols notably impact flavour and also act as the precursors for ester formation [56]. They are closely related to protein synthesis and amino acid assimilation [50],

and their final amount in beer can be altered by fermentation temperature [57]. Increased amounts of higher alcohols compared to other VOCs were detected in all three beers (Table 7). The beer fermented with *Hyb1CIT1* contained the highest amounts of 3-methyl-1-butanol, 2-methyl-1-propanol and 2-methyl-1-butanol among the other beers; however, their concentrations were far below the threshold levels [55]. Comparing the results with a similar study of the inter-specific hybrid of *S. mikatae* and commercial wine fermenting *S. cerevisiae*, hybrids were able to form greater amounts of higher alcohols and esters than in this work [23]. Even though the hybrids of *S. mikatae* are known to contribute to the aromatic profile of wine, the level of volatile compounds is strictly dependent on the number of fermentable sugars, which is limited during the production of non-alcoholic beers [23]. The formation of 4-vinylguaiacol from ferulic acid during boiling or yeast fermentation is often related to an unpleasant smoky/bacon aroma [58]. Its threshold concentration in beer is 0.2–0.3 mg L^{-1} [35] and was slightly exceeded in this study in all three non-alcoholic beers (Table 7) but had no impact on the beer flavour.

3.8. Principal Component Analysis

The scatter plot (Figure 5a) with principal components 1 and 2 revealed that 47.08% of the variation in the data making up the beer VOC's profile (Table 7) is explained by first two principal components. Beers fermented with *S. mikatae* (*Sm*) are situated on the lower left side of the scatter plot; the beers *CIT1* are situated on the right lower side of the scatter plot. The beers fermented with hybrid (*Hyb1CIT1*) are situated on the upper right and left side of the scatter plot. We can claim that the real hybrid inherited both parental features from VOCs profiles, but as we can see in Table 7, no marked differences were noted among the VOC profiles of the non-alcoholic beers. However, *Hyb1CIT1* beers were characterised by higher amounts of higher alcohols, namely, 3-methyl-1-butanol, 2-methyl-1-propanol and 2-methyl-1-butanol. This analysis provided us with information about novel hybrids and fermentation similarities with parental strains of the maltose-negative mutant *S. cerevisiae* and wild-type *S. mikatae*.



(**a**) (**b**)

Figure 5. Scatter plot (**a**) and loading plot (**b**) of the principal component analysis (PCA) of VOCs profiles of beer samples. Beer sample abbreviations (*Hyb1CIT1*, *Sm* and *CIT1*) correspond to yeast abbreviations (Table 1) used in beer production.

4. Conclusions

This work provided a clearer insight into the breeding of hybrid *Saccharomyces* species crossed between mutant strains of *S. cerevisiae*, with higher organic acid production, and wild-type *S. mikatae* targeted for non-alcoholic beer production. Both parental strains as well as six hybrid strains were tested for osmotolerance and ability to propagate in 30% (*w*/*w*) glucose solution. Almost identical growth curves of maltose-negative hybrids were obtained. However, in comparison with parent strains, lower absorbance (A_{600nm})

values were obtained for the hybrids. PCR analysis confirmed that one hybrid, *Hyb1CIT1*, inherited recombinant mtDNA from both parents and was highlighted for further use. Final beer production by fermenting 7°P wort with this hybrid and its parents was conducted, and the organoleptic profile of beers was determined. We successfully produced non-alcoholic beers with $\leq$0.05% (*v*/*v*) of ethanol using both hybrid as well as parental strains. The beer produced with hybrid *Hyb1CIT1* contained a similar content of organic acids as the beer produced with the parental strain *S. cerevisiae CIT1*, but the production of an undesirable amount of acetic acid was successfully eliminated. The aromatic profile of beer fermented with hybrid yeast *Hyb1CIT1* was neutral, with no negative off-flavours. Even though the hybrid *Hyb1CIT1* was able to produce slightly higher concentrations of 3-methyl-1-butanol, 2-methyl-propanol and 2-methyl-1-butanol than the parental strains *S. mikatae* and *S. cerevisiae*, these concentrations were negligible in terms of flavour. The data for volatile organic compounds were analyzed by principal component analysis, which confirmed no significant differences between parental strains and their hybrid offspring. This work could be of help in understanding the approach of specific hybrid breeding targeted for non-alcoholic beer production. The maltose-negative parental strain *S. mikatae*, which was used for non-alcoholic beer production for the first time, implemented a neutral aromatic profile into the beer and could be further used in co-fermentations with other flavour-inducing yeast strains.

Author Contributions: Conceptualisation, P.V., P.S. and D.Š.; Methodology, P.V.; Software, P.V.; Validation, P.V.; Formal Analysis, P.V.; Investigation, P.V. and P.S.; Resources, P.S., T.K., D.Š. and P.D.; Data Curation, P.V. and Z.R.; Writing—Original Draft Preparation, P.V.; Writing—Review and Editing, Z.R.; Visualisation, P.V. and Z.R.; Supervision, D.Š. and P.D.; Project Administration, D.Š. and P.D.; Funding Acquisition, P.S., D.Š. and P.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences VEGA 1/0013/20, by the Slovak Research and Development Agency APVV-15-0333, and with the support of the Ministry of Education, Science, Research and Sport of the Slovak Republic within the Research and Development Operational Programme for the project 'University Science Park of STU Bratislava', ITMS 26240220084.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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