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Modifying flavor profiles of *Saccharomyces spp.* for industrial brewing using FIND-IT, a non-GMO approach for metabolic engineering of yeast

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ABSTRACT

Species of *Saccharomyces* genus have played an irreplaceable role in alcoholic beverage and baking industry for centuries. *S. cerevisiae* has also become an organism of choice for industrial production of alcohol and other valuable chemicals and a model organism shaping the rise of modern genetics and genomics in the past few decades. Today's brewing industry faces challenges of decreasing consumption of traditional beer styles and increasing consumer demand for new styles, flavors and aromas. The number of currently used brewer's strains and their genetic diversity is yet limited and implementation of more genetic and phenotypic variation is seen as a solution to cope with the market challenges. This requires modification of current production strains or introduction of novel strains from other settings, e.g. industrial or wild habitats into the brewing industry. Due to legal regulation in many countries and negative customer perception of GMO organisms, the production of food and beverages requires non-GMO production organisms, whose development can be difficult and time-consuming. Here, we apply FIND-IT (Fast Identification of Nucleotide variants by DigITal PCR), an ultrafast genome-mining method, for isolation of novel yeast variants with varying flavor profiles. The FIND-IT method uses combination of random mutagenesis, droplet digital PCR with probes that target a specific desired mutation and a sub-isolation of the mutant clone. Such an approach allows the targeted identification and isolation of specific mutant strains with eliminated production of certain flavor and off-flavors and/or changes in the strain metabolism. We demonstrate that the technology is useful for the identification of loss-of function or gain of function mutations in unrelated industrial and wild strains differing in ploidy. Where no other phenotypic selection exists, this technology serves together with standard breeding techniques as a modern tool facilitating a modification of (brewer's) yeast strains leading to diversification of the product portfolio.

1. Introduction

Yeasts have been accompanying human civilizations for thousands of years. Yeasts of the *Saccharomyces* genus, particularly the model organism *S. cerevisiae* have been used in food and beverage production using its natural unique capabilities to efficiently ferment sugars to alcohol and CO_2 [1,2]. More recently the yeast has also become widely used in industrial biotechnology for production of fuels from renewable biomass due to its robustness, stress resistance and high alcohol tolerance [1,3]. Deep knowledge and understanding of its genetic and physiological properties and existence of genetic engineering tools made it possible to establish the organism as a cell factory for production of wide range of added value chemicals [4,5]. Emergence and development of advanced gene editing tools [6,7] has created a space for more efficient metabolic engineering thus broadening the substrate and product portfolio of yeast based cell factories even further [8,9].

Abbreviations: 4-VG, 4-vinyl guaiacol; dPCR, digital polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction; qPCR, quantitative polymerase chain reaction; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9; FIND-IT, Fast Identification of Nucleotide variants by droplet DigITal PCR; GM, genetic modification; GMO, genetically modified organism; HO, homothallic switching endonuclease; MCFA, medium chain fatty acids; POF, phenolic off-flavor; RDF, real degree of fermentation.

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In contrast, the brewing industry is a rather conservative one. Brewing process and product development have not changed much over the decades despite all technological advances. Brewer's yeasts ale and lager also known as the top-fermenting and bottom-fermenting yeasts, are the two main types of yeast used nowadays [10]. Despite remarkable natural genetic diversity of yeasts [11], the number of yeast strains used commercially is very limited [12]. However, with the emergence of craft brewing and increasing consumer demand for new beer styles and tastes also big brewers realized that yeast contributes significantly to the beer quality and aroma [13]. There is a room for modification and improvement of currently used production strains and introduction of novel strains or non-conventional yeast species bringing more flavor diversity into the product portfolio [14]. Although there has been ongoing intensive research of brewer's yeast, the development of new strains is hampered by their complicated nature such as complex (often hybrid, aneuploid and polyploid) genomes and resulting difficulties in classical breeding [15]. Moreover, the food industry is reluctant to use recombinant DNA technologies, resulting in generation of genetically modified organisms (GMO) and relies on conventional methodology such as random mutagenesis, breeding or adaptive evolution [16,17]. This is driven by strict regulatory rules for GM food especially in the EU but mainly by a negative public perception of genetic modifications, and therefore a potential negative influence on the company reputation. Advanced gene editing technologies have been successfully established in industrial yeast [18] and also lager yeast strains [19]. Moreover, a potential of GM yeast strains for brewing has been shown [20]. It resulted in a roll out of Lallemand's Sourvisiae® and the use of GM *S. cerevisiae* strains for production of beers with increased concentration of volatile thiols by a few craft breweries across the US [21]. However, the widespread use of GMOs in the food and beverage industry and corresponding legal regulations are still a subject of future societal and scientific debate [22–24].

Digital PCR (dPCR) is a method that similarly to quantitative PCR (qPCR) allows to amplify and quantify a specific target DNA sequence. Droplet dPCR (ddPCR) is a variant of this method that allows for simple partitioning of the sample, where every droplet is a micro PCR reactor. The droplets are then analyzed individually by a mechanism similar to flow cytometry [25]. It provides absolute quantification with higher accuracy when compared to qPCR, does not require a standard curve and is less sensitive to the sample impurity [26]. Apart from its application in copy number variation [27] and gene expression studies [28] it has become a valuable tool in clinical diagnostics [29]. Here it enables the quantification of a specific (mutant) DNA sequence that is only present in low copy numbers within an abundant wild type sequence population in the sample. The rare mutation detection screening has been widely used in oncology to study genetic alterations in a wide variety of cancers [30]. More recently, a method designated as FIND-IT (Fast Identification of Nucleotide variants by DigITal PCR) employing ddPCR has also been used for the identification of particular mutants in mutated seed libraries in plants and also cell populations of microorganisms, including yeast [31,32]. The FIND-IT method relies on three pillars: (i) generation of a library of genetic variants such as populations generated by e.g. chemical mutagenesis, (ii) sensitive ddPCR technology that allows for (iii) sample pooling and splitting. This enables the pooling of hundreds or more variants before DNA isolation. The use of the three components expands the size of libraries to be screened to hundreds of thousands of variants [31].

In this study, we explore the FIND-IT technology to identify and isolate yeast mutants carrying specific point mutations in large yeast populations. The method enables the identification of both mutants with gene inactivation (loss of function) or gain of function mutations in various unrelated yeast strain backgrounds. Using this technology, we generated derivatives of a non-brewer's yeast strain with particular changes in flavor production as well as derivatives of production lager yeasts showing complex changes in their flavor profiles. Moreover, we show isolation of a heterothallic variant of the wild *S. eubayanus* isolate [33] that should facilitate the breeding and *de novo* creation of lager strains bringing more phenotypic diversity into the lager yeast strain portfolio. The strains obtained by the described method are exempt from the obligations of the EU GMO Directive [34] and the method is therefore beneficial where GM techniques are undesired. We demonstrate that FIND-IT serves as a tool to isolate specific mutants in cases where the options of selection for a particular phenotype or high throughput screening phenotypic assays are limited.

2. Materials and methods

2.1. Strains and media

All strains used in this study are listed in Table 1. Strains were grown in standard YPD (1 % yeast extract, 2 % peptone, 2 % glucose) or SD (0.67 % yeast nitrogen base without amino acids, 2 % glucose) at various temperatures based on the particular purpose. For solid media 2 % agar was added. Sporulation (SPOR) medium contained 1 % potassium acetate, 0.1 % yeast extract and 0.1 % glucose. For certain experiments glucose was replaced with 2 % galactose (YPG and SG) and 200 µl of X-α-gal (5 mg/ml in dimethylformamide) was spread in on agar plates. For fermentation experiments a wort was prepared from 70 % Pilsner malt and 30 % barley grist, which was mashed in 2.7 l water/kg of grain. The mashing profile contained an initial mashing step at 50 °C for 30 min, followed by a temperature increase to 65 °C for 50 min. The mashing was stopped at 78 °C for 5 min. The extract concentration in the final wort was 16°P.

2.2. Growth and fermentation conditions

Yeast microscale starter cultures were grown in Greiner 96-well microtiter plates in 150 µl YPD medium. 10 µl of the overnight culture were transferred into 96-well square deep-well plates filled with 240 µl of wort. The plates were covered with anaerobic sandwich covers and grown in the Growth profiler instrument (Enzyscreen) at 16 °C with 190 rpm shaking speed. The cameras were set to capture an image of the plates every 30 min. To observe biomass accumulation the images were analyzed by a software provided by the manufacturer. Screening for phenolic off-flavor (POF^-) mutants was performed by fermentation in 96-square deep-well plates in YPD supplemented with 1 mM ferulic acid and measuring the supernatant absorbance at 325 nm using a UV spectrophotometer (Tecan) [35]. For large scale fermentations 3 ml of overnight YPD cultures were inoculated into 30 ml of wort in 100 ml shake flasks with foam plug and grown for 2 more days. Cell titer of the resulting starter culture was determined using Cellometer (Nexcellom Biosciences) according to the manufacturer's instructions, and cells were transferred into standard 250 ml glass bottles together with fresh wort in a total volume of 160 ml to a final cell concentration of 1–1.5 × 10^7 cells per ml. The fermentations were carried out anaerobically with stirring and gas production monitored using the Ankom RF gas production system (Ankom Technology) at 16 °C unless indicated otherwise [36]. After six days liquid samples were collected by centrifugation and analyzed using analytical methods.

2.3. Sporulation, breeding and hybrid selection

To sporulate yeast strains, fresh overnight cultures were patched onto SPOR plates, which were incubated at room temperature for several days. The plates were periodically inspected under a microscope for the formation of asci. To release the ascospores, asci were treated in 50 µl of Zymolyase (US Biological) solution (0.5 mg/ml) for 20 min at 30 °C with moderate shaking. 400 µl of sterile water was added and the treated asci were dropped onto an YPD plate. Spores were isolated using a dissection microscope (MSM400, Singer Instruments). Mating type was determined using a pheromone response halo assay on YPD plates containing 0.05 % Triton X-100 and covered with a lawn of one of two

Table 1
List of strains used in the study.

strain name	organism	description	genotype	source
PE-2	*S. cerevisiae*	bioethanol strain	*MATa/MATα*	Basso et al., 2008
Danstar Windsor ale	*S. cerevisiae*	top-fermenting yeast, Lallemand Windsor Danstar	*unknown*	Maltbazaren (www.maltbazaren.dk)
Ale yeast	*S. cerevisiae*	top-fermenting yeast	*unknown*	Carlsberg Research Laboratory
Lager yeast	*S. pastorianus*	bottom-fermenting yeast	*unknown*	Carlsberg Research Laboratory
S. eubayanus CBS12357	*S. eubayanus*	wild yeast	*MATα/MATα*	Libkind et al., 2011
S.c. PE-2 a	*S. cerevisiae*	PE-2 spore	*MATa*	This study
S.c. PE-2 α	*S. cerevisiae*	PE-2 spore	*MATα*	This study
S.c. PE-2 a fdc1	*S. cerevisiae*	POF- spore clone derived from breeding of S.c. PE-2 a S.c. PE-2 α fdc1 spores and subsequent sporulation	*MATa* $fdc1^{G513A}$	This study
S.c. PE-2 α fdc1	*S. cerevisiae*	mutagenized PE-2 α, POF^- spore	*MATα* $fdc1^{G513A}$	This study
S.c. PE-2 fdc1	*S. cerevisiae*	hybrid of S.c. PE-2 a fdc1 and S.c. PE-2 α fdc1, POF^-	*MATa/MATα* $fdc1^{G513A}$/$fdc1^{G513A}$	This study
S.c. PE-2 α iah1	*S. cerevisiae*	mutagenized PE-2 α, $iah1^-$ spore	*MATα* $iah1^{G386A}$	This study
S.c. PE-2 a iah1 fdc1	*S. cerevisiae*	PE-2 POF^- spore	*MATa* $iah1^{G386A}$ $fdc1^{G513A}$	This study
S.c. PE-2 α iah1 fdc1	*S. cerevisiae*	POF^-, $iah1^-$ spore clone derived from breeding of S.c. PE-2 a fdc1 S.c. PE-2 α iah1 spores and subsequent sporulation	*MATα* $iah1^{G386A}$ $fdc1^{G513A}$	This study
S.c. PE-2 fdc1 iah1/IAH1	*S. cerevisiae*	hybrid of S.c. PE-2 a iah1 fdc1 and S.c. PE-2 α fdc1	*MATa/MATα* $iah1^{G386A}$/*IAH1* $fdc1^{G513A}$/$fdc1^{G513A}$	This study
S.c. PE-2 fdc1 iah1	*S. cerevisiae*	hybrid of S.c. PE-2 a iah1 fdc1 and S.c. PE-2 α iah1 fdc1	*MATa/MATα* $iah1^{G386A}$/$iah1^{G386A}$ $fdc1^{G513A}$/$fdc1^{G513A}$	This study
Windsor a spore	*S. cerevisiae*	sporulation of Lallemand Windsor Danstar	*unknown behaves as MATa*	This study
Ale a spore	*S. cerevisiae*	sporulation of Ale yeast	*unknown behaves as MATa*	This study
lager spore a	*S. pastorianus*	sporulation of lager yeast	*unknown behaves as MATa*	Carlsberg Research Laboratory
lager spore α	*S. pastorianus*	sporulation of lager yeast	*unknown behaves as MATα*	Carlsberg Research Laboratory
Ale a spore fdc1/FDC1	*S. cerevisiae*	mutagenized Ale a spore	*unknown* $fdc1^{G513A}$/*FDC1*	This study
PE-2 x ale	*S. cerevisiae*	hybrid of S.c. PE-2 α fdc1 and Windsor a spore	*unknown*	This study
PE-2 x lager	*S. pastorianus*	hybrid of S.c. PE-2 a fdc1 and lager spore α	*unknown*	This study
lager Tyr1 A184T	*S. pastorianus*	mutagenized lager yeast	*unknown* $TYR1^{G550A}$/*TYR1*/*TYR1*/*TYR1*	This study
lager Tyr1 A188T	*S. pastorianus*	mutagenized lager yeast	*unkown* $TYR1^{G562A}$/*TYR1*/*TYR1*/*TYR1*	This study
lager Tyr1 T210I	*S. pastorianus*	mutagenized lager yeast	*unknown* $TYR1^{C629T}$/*TYR1*/*TYR1*/*TYR1*	This study
S. eubayanus ho/HO	*S. eubayanus*	mutagenized S. eubayanus CBS12357	*SeMATa/MATα SeHO/Seho*^{C238T}	This study
S.e. ho a	*S. eubayanus*	HO^- a S.e. spore, sporulation of S. eubayanus ho/HO	*SeMATa* $Seho^{C238T}$	This study
S.e. ho α	*S. eubayanus*	HO^- α S.e. spore, sporulation S. eubayanus ho/HO	*SeMATα* $Seho^{C238T}$	This study
S.e. fdc1 a	*S. eubayanus*	mutagenized S.e. ho a POF^- spore	*SeMATa* $Seho^{C238T}$ $Sefdc1^{G477A}$	This study
S.e. fdc1 α	*S. eubayanus*	mutagenized S.e. ho α POF- spore	*SeMATα* $Seho^{C238T}$ $Sefdc1^{G477A}$	This study
Novel lager 1 (NL1)	*S. cerevisiae x S. eubayanus*	hybrid of S.c. PE-2 a and S.e. ho α	*MATa/SeMATα* $Seho^{C238T}$	This study
Novel lager 2 (NL2)	*S. pastorianus spore x S. eubayanus*	hybrid of lager spore a and S.e. ho α	*unknown/SeMATα* $Seho^{C238T}$	This study
Novel lager 3 (NL3)	*S. cerevisiae x S. eubayanus*	hybrid of S.c. PE-2 a fdc1 and S.e. fdc1 α	*MATa/SeMATα Scfdc1*^{G513A} $Seho^{C238T}$ $Sefdc1^{G477A}$	This study
Novel lager 4 (NL4)	*S. pastorianus spore x S. eubayanus*	hybrid of lager spore a and S.e. fdc1 α	*unknown/SeMATα* $Seho^{C238T}$ $Sefdc1^{G477A}$	This study
α-PS	*S. cerevisiae*	S.c. BY4741 α pheromone sensitive strain	*MATa sst2Δ*	Euroscarf
a-PS	*S. cerevisiae*	S.c. BY4742 a pheromone sensitive strain	*MATα sst2Δ*	Euroscarf

mating pheromone sensitive (PS) strains [37]. The growth inhibitory zone of the PS strains around a tested strain was visually scored after 2 days of growth. Genetic crossing was performed by standard techniques as described in [38]. Briefly, overnight cultures of equal concentrations were mixed and patched on YPD plates. After approximately 12 hrs the mixed culture was diluted and plated on agar plates according to the respective selection process. For detection of *S. eubayanus* colonies and hybrids agar plates with galactose and X-α-gal. Some yeast species and strains, such as e.g. *S. eubayanus* possess genes encoding enzymes with α-galactosidase activity [39]. Such genes are regulated by the GAL genes, whose expression is triggered by the presence of galactose. Alpha-galactosidase converts the colorless X-α-gal to the blue product. For the selection of hybrids with a lager spore, SD (or SG X-α-gal) plates with metsulfuron-methyl (10 mg/ml) were used. Resistance to this compound is driven by dominant mutations in *ILV2* gene resulting in an impaired function of the acetolactate synthase enzyme [40,41]. Metsulfuron-methyl can therefore be used as a selection for mutants (the lager spore) and their derivatives (hybrids). Identification of *S. cerevisiae* hybrids was done by replica plating of colonies on YPD plates onto SPOR plates. Colonies with sporulating cells were considered as hybrids.

2.4. Mutagenesis conditions

Strains of interest were grown in 3 ml of YPD medium in 12-ml culture tubes overnight. The cells were collected by centrifugation and washed twice with PBS + 1 mM EDTA buffer. The cell number was determined by Cellometer (Nexcellom Biosciences) according to the manufacturer's instructions. 10^8 cells in 1 ml of the buffer were used and

30 µl of EMS was added into each tube [42]. The length of EMS mutagenesis was optimized for each strain whereat a survival rate between 45–65 % was considered optimal (Table S1). The efficiency of the mutagenesis procedure was evaluated by plating EMS treated and non-treated cultures on plates with canavanine (120 mg/l) as described in [42]. The mutation load was increased by two orders of magnitude using the procedure as shown in the original protocol [42].

2.5. Library preparation and digital PCR analysis

The FIND-IT methodology was applied to target a desired mutation in each yeast consisting of a mutagenesis, digital PCR step and a sub-isolation steps [31,43]. The strains of interest were grown overnight. The cells were treated and mutagenized as described above. As an optional step multiple successive rounds of mutagenesis with or without selection after each round were performed in order to potentially increase the mutation load in the population and thus the chance of successful mutant identification. After the treatment cells were aliquoted into a 96-well plate in the concentration of approximately 2500 viable cells per well (taking into account the survival ratio of the population after the treatment). Thus the cell library consisted of approximately 2×10^5 viable cells in total. The cultures were grown overnight to reach late exponential/early stationary phase culture. DNA was isolated using MasterPure™ Yeast DNA Purification Kit (Lucigen). Digital PCR technology was used for identification of a specific mutation in the mutagenized population. TaqMan PCR probes and primers were designed using the Bio-Rad design tool (Bio-Rad) to distinguish between two different nucleotides at the same location in a targeted gene of interest (Table S2). A master mix of the TaqMan assay solution, DNA and PCR reagents (Bio-Rad SuperMix) was separated into droplets using the QX200 AutoDG Droplet Digital PCR system (Bio-Rad), according to the manufacturer's instructions. After the PCR reaction, the droplets were analyzed using the QX200 instrument (Bio-Rad). The difference in abundance between two different nucleotides at the same position in a gene of interest was calculated based on the amplitude of the signals of the TaqMan assays using the software analysis tool Quantasoft (Bio--Rad). Population in a well with identified mutation was grown for 6 hrs in an Eppendorf tube with 0.5 ml YPD medium with shaking. The cell culture was diluted and aliquoted in the final concentration of 50–100 cells per well and analyzed as described above. Finally, the culture from the well with the present mutation was plated on YPD plates and 96 colonies randomly picked into a microtiter plate using the QPix 400 colony picker (Molecular Devices). Subsequently, DNA was isolated from the cultures grown overnight and the mutation presence verified by ddPCR.

2.6. Analytical methods

Gas chromatography was used to determine the concentration of volatile compounds in the samples. The main flavor active volatiles and their precursors [13,44] included alcohols (propanol, isobutanol, isoamyl alcohol, 2-phenylethanol), acids (caproic acid, caprylic acid, capric acid), esters (ethyl acetate, isobutyl acetate, isoamyl acetate, ethyl caproate, ethyl caprylate, ethyl butyrate and 2-phenylethyl acetate) and phenols (4-vinylguaiacol). The volatiles were extracted from wort samples with carbon disulphide (CS_2) [45]. The subsequent qualitative analysis was performed using gas chromatography with a flame ionization detector (GC-FID). An Agilent 6890 A gas chromatograph with a split/splitless injector and a 30 m x 0.32 mm× 0.25 µm DBWAX capillary column was used for the analysis. The sample extract was introduced to the preheated injector at 250 °C, where it was volatilized and led to the column with helium as carrier gas. In the flame ionization detector, the components were led through an air-hydrogen flame at 250 °C that decomposed the component due to the high temperature. For determination of diketones (diacetyl) head space gas chromatography with electron capture detection (HS GC-ECD) was used. A gas chromatograph with a head space injector, a CP-Wax 60 m x 0.25 mm × 0.39 mm capillary column and an electron capture detector with nickel-63 as β-emitter was used for the analysis. The head space samples were collected from the sample vials by the head space injector at 105 °C as small amounts of head space gas and introduced to the column. The head space here is defined as the gas between the sample surface (head) and the top of the closed vial. An argon/methane mixture was used as carrier gas. Data analysis was performed using Chemstation software. Alcohol concentrations and real degree of fermentation (RDF) were measured according to the manufacturer's instructions using the Alcolyzer Beer Analyzing System consisting of the DMA 4500 M density meter and an Alcolyzer Beer ME measuring module (Anton Paar).

3. Results

3.1. FIND-IT method allows for identification of specific mutations in different yeast strain backgrounds

We explored the potential of FIND-IT technology and standardized the experimental set up for finding the mutation of choice in cell libraries of various yeast strains (Fig. 1). It covers all steps from the library preparation (Fig. 1A), over mutant identification to the final mutant isolation from the population (Fig. 1B, for details see also Materials and methods section). As a proof of concept, a haploid spore of the bioethanol strain PE-2 (Table 1) was chosen. The strain is heterothallic [46], has an excellent fermentation performance at a wide range of temperatures and a proven record of use in industrial settings [47]. Moreover, wild non-brewer's *Saccharomyces* strains often produce 4-VG (considered as undesired clove-like flavor in majority of beer styles) by enzymatic conversion of ferulic acid in wort [11,48]. Two genes (*FDC1*, *PAD1*) encode for enzymes responsible for the conversion and inactivation of either of these lead to the lack of the decarboxylase activity [49] and therefore no production of 4-VG. Thus, there is a clear link between the particular genotype and observable phenotype. The population of a haploid PE-2 spore (S.c. PE-2 α) was mutagenized and screened for the presence of a nonsense mutation in the *FDC1* gene. A specific G to A transition at position 513 would introduce a premature STOP codon in the coding sequence. Two mutagenized libraries were prepared, both with two consecutive rounds of mutagenesis. For one of the libraries, the first mutagenized population was plated on canavanine containing plates and canavanine-resistant cells were used for the subsequent second round of mutagenesis. The second library was only recovered in liquid YPD for several hours after the first mutagenesis. The G513A mutation could be identified in both libraries, yet with higher frequency in the population when the canavanine selection was applied (Fig. 2A). Since the mutation could be identified in both libraries, the mutagenesis protocol without any selective step was subsequently used as a standard approach to simplify and speed up the method and reduce the risk of acquiring too many potential off-target mutations through the selection process.

A haploid *fdc1* mutant (S.c. PE-2 α fdc1) was subsequently isolated from the library and its phenotype confirmed by fermentation in ferulic acid containing medium and absorbance measurement at 325 nm [35]. An a POF^- spore (S.c. PE-2 a fdc1) was prepared by breeding of the mutagenized spore with a wild type a spore (S.c. PE-2 a) and subsequent sporulation, tetrad dissection and selection of spores with the POF^- phenotype (Fig. S1). A diploid POF^- PE-2 strain (S.c. PE-2 fdc1) was bred by crossing the a and α mutant spores. The POF^- phenotype was confirmed for all strains by fermentation in wort at 16 °C. Both haploid and diploid mutants showed absence of significant amounts of 4-VG production while other relevant characteristics such as RDF or production of other flavor-active compounds remained similar to the wild type strains (Fig. 2A). The versatility of the described approach was proven by the identification of the same G513A mutation in a mutant library of a spore clone derived from a brewer's yeast strain (Ale yeast, Table 1). However, even after several rounds of restreaking, the final isolate still

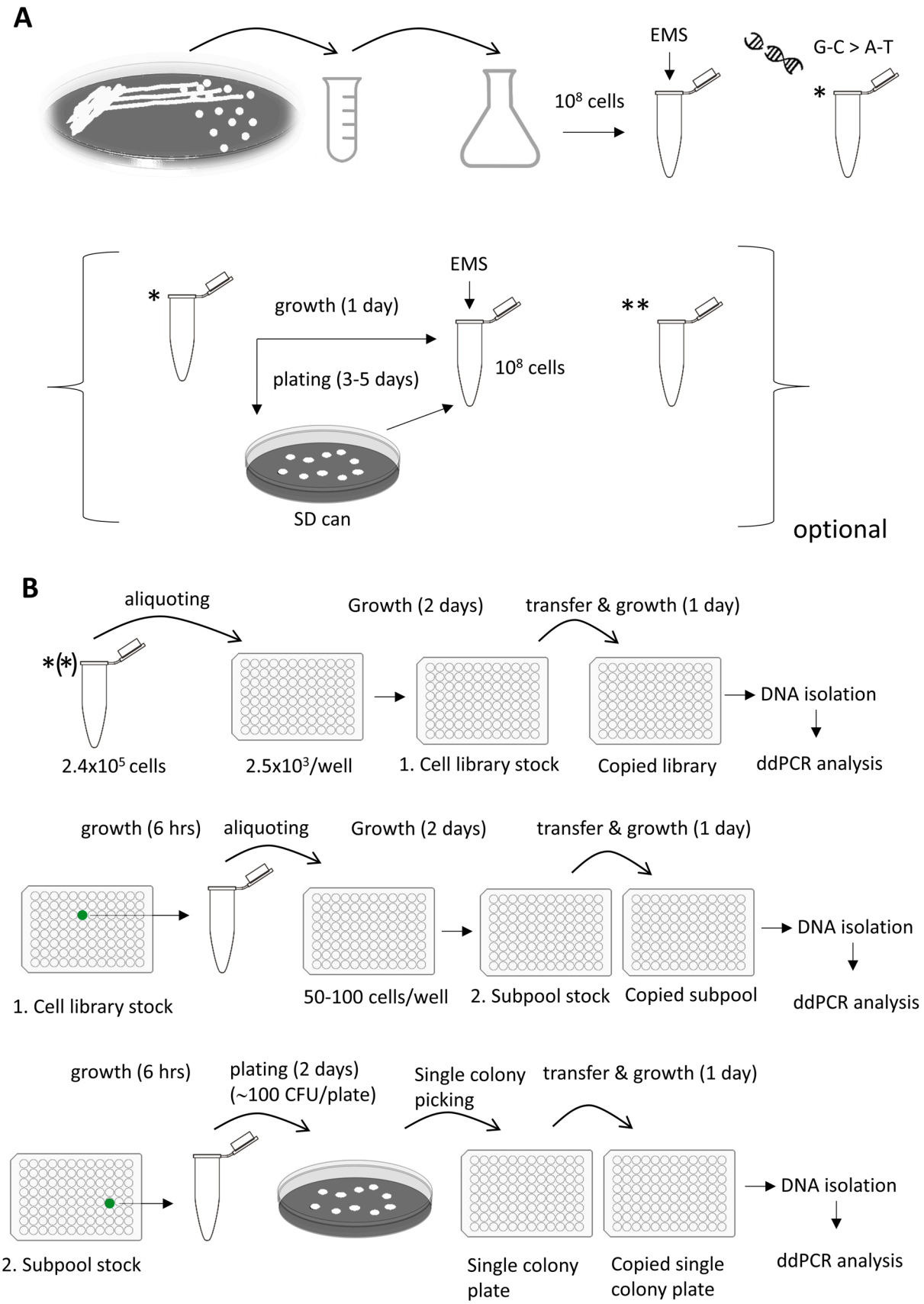

Fig. 1. Schematic illustration of the FIND-IT method for identification and isolation of specific mutants from a mutagenized yeast population. A) Preparation of mutagenized libraries. B) Subpooling and isolation of particular mutants.

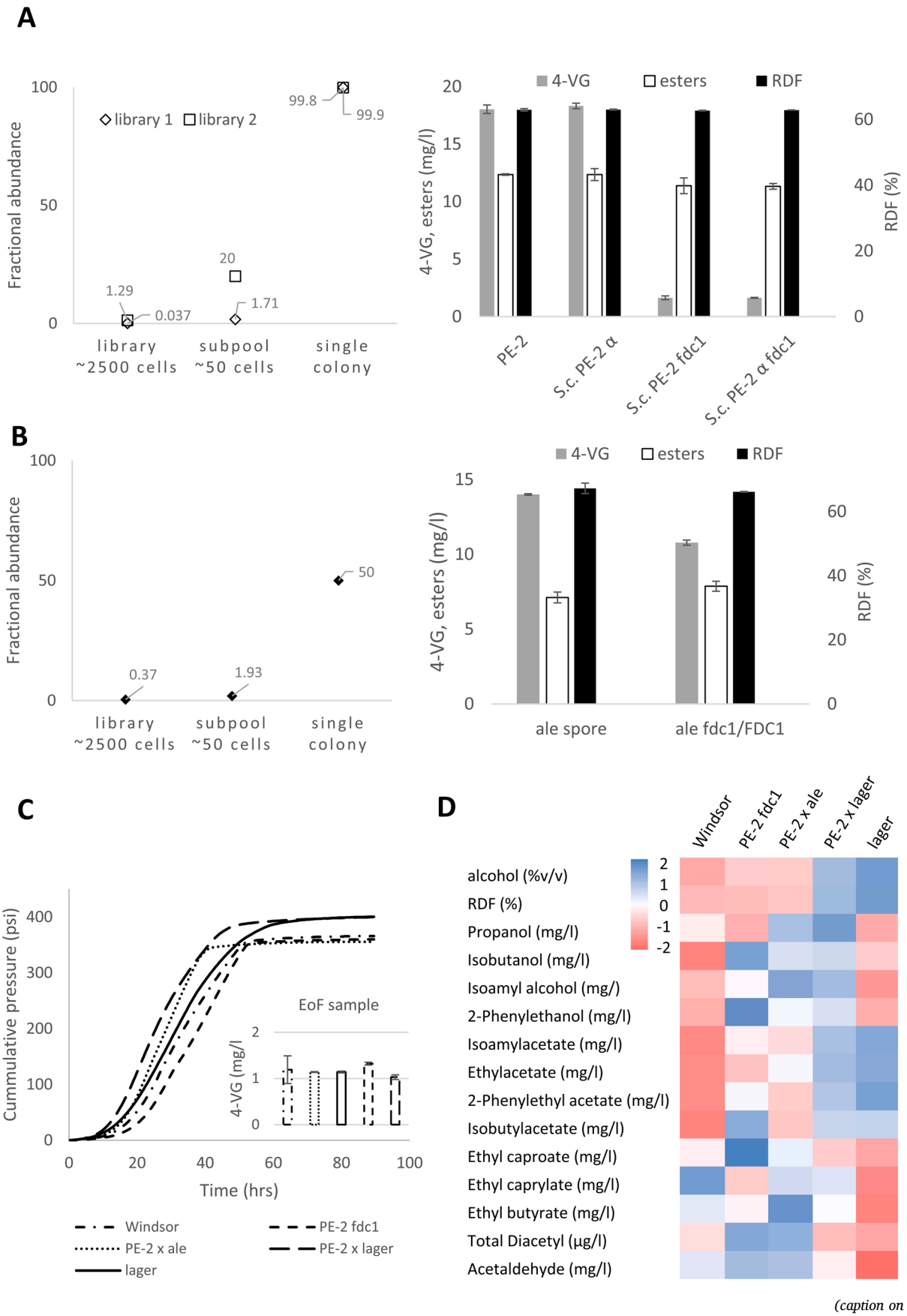

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Fig. 2. Isolation of *FDC1* mutants of *S. cerevisiae* strains and development of hybrids derived thereof. A) Isolation of POF$^-$ mutants of a bioethanol strain. Left, the graph of fractional abundance shows the frequency of the *FDC1*^{G513A} mutation in the population of the original library (~2500 cells), subpool (~50 cells) and single colony. Libraries were made using two consecutive rounds of mutagenesis, with library 1 using no selection and library 2 using SD canavanine selection plates after initial EMS treatment. Right, the graph shows 4-VG, ester production and RDF (secondary y axis) of PE-2 strain, haploid spore (S.c. PE-2 α) and *fdc1* mutants (haploid – S.c. PE-2 α fdc1 and diploid – S.c. PE-2 fdc1) in finished wort fermentations. The values are average of 3 replicates. Error bars represent standard deviation (N = 3). B) Isolation of *FDC1* mutants of an ale strain. Left, the graph of fractional abundance shows the frequency of the *FDC1*^{G513A} mutation in the population of the original library (~2500 cells), subpool (~50 cells) and single colony. Right, the graph shows 4-VG, ester production and RDF (secondary y axis) of Ale a spore (ale spore) and its *fdc1* (ale fdc1/FDC1) mutant in finished wort fermentation. C) Left, cumulative pressure graph demonstrates the course of fermentation of Windsor ale (Windsor), PE-2 POF$^-$ (PE-2 fdc1), lager yeast (lager) and their hybrids (PE-2 x ale, PE-2 x lager). Right, chart displays concentration of 4-VG in end of fermentation samples (EoF) of all the strains and hybrids. The values are average of 3 replicates. Error bars represent standard deviation (N = 3). D) Flavor profiles of the strains. Color coding is based on a Z-score calculated from absolute values in the data set (absolute numbers are in Table S3). The color scale is displayed.

showed a 50 % fractional abundance of the mutation in the population suggesting that there was a wild type copy of the gene still present in the genome (Fig. 2B). Even though the 4-VG level was lower in the finished fermentations of the mutant strain when compared to the wild type spore, it was still significantly above the sensory threshold (Fig. 2B). A higher genome complexity (aneuploidy or polyploidy) is relatively common among brewer's yeast strains even in spore clones [11,50]. Thus, further mutagenesis and/or breeding would be necessary to observe the full POF- phenotype.

To demonstrate the applicability of the POF$^-$ mutant spore of the bioethanol strain in a brewer's yeast breeding program, the strain was crossed with a spore derivative of the Danstar Windsor ale yeast (Windsor a spore, Table 1). The resulting hybrid fermented faster than both parental strains with apparently more efficient maltose fermentation than the PE-2 strain (Fig. 2C). It also combined rather neutral Windsor flavor profile at the given conditions with more PE-2 specific features such as higher production of heavy alcohols and fatty acid derived esters (Fig. 2D, Table S3). The POF$^-$ PE-2 derivative was also

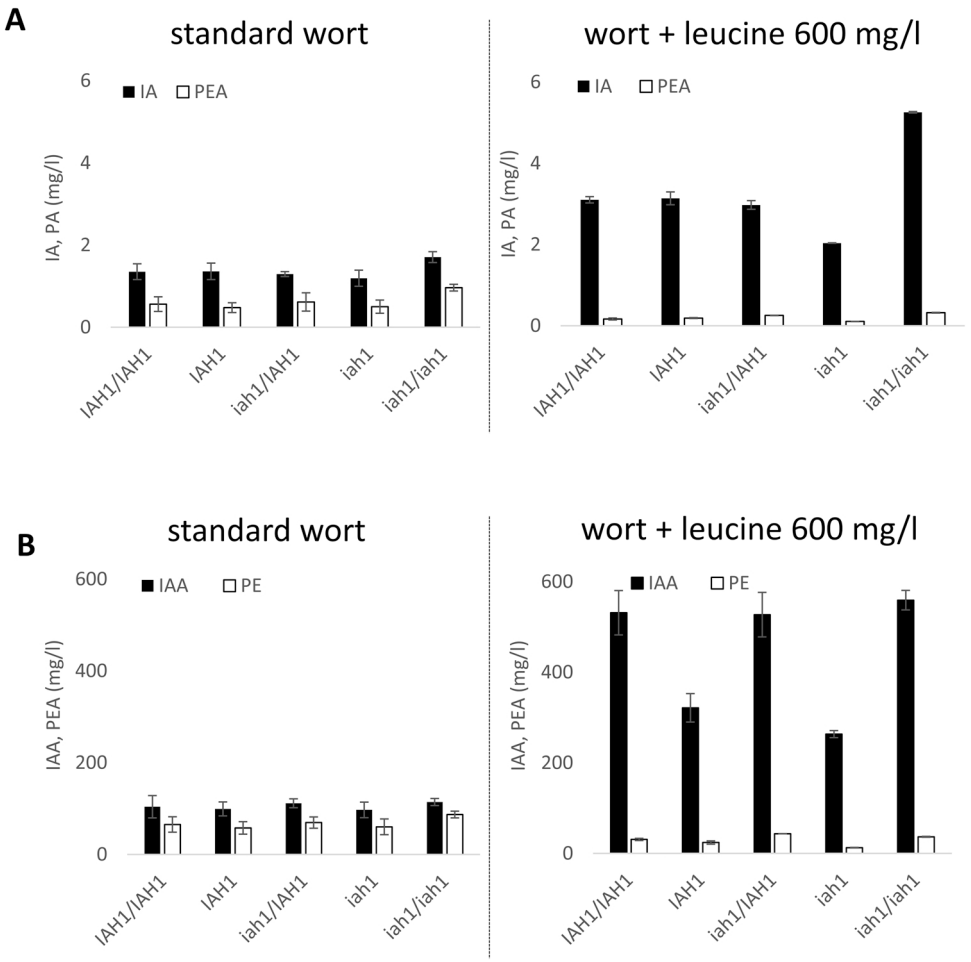

Fig. 3. Identification of *IAH1* mutation in various strains. A) The graphs show concentration of isoamyl acetate (IA) and 2-phenylethyl acetate (PEA) in finished wort fermentations of PE-2 strain (IAH1/IAH1), its haploid spores (IAH1) and their derivatives with non-sense mutation in *IAH1* gene (haploid – iah1, diploid with one mutated allele – iah1/IAH1, diploid with both mutated alleles – iah1/iah1). For PE-2 strain and PE-2 iah1/IAH1 the values are average of 3 replicates. Error bars represent standard deviation (N = 3). For haploid spores and diploid mutant (iah1/iah1) the values are average of 4 different spores/clones of the designated genotype run in 3 replicates each. Left, standard wort conditions, right, wort supplemented with leucine. B) The graphs show concentration of isoamyl alcohol (IAA) and 2-phenylethanol (PE) in finished wort fermentations of PE-2 strain (IAH1/IAH1), its haploid spores (IAH1) and their derivatives with non-sense mutation in *IAH1* gene (haploid – iah1, diploid with one mutated allele – iah1/IAH1, diploid with both mutated alleles – iah1/iah1). For PE-2 strain and PE-2 iah1/IAH1 the values are average of 3 replicates. Error bars represent standard deviation (N = 3). For haploid spores and diploid mutant (iah1/iah1) the values are average of 4 different spores/clones of the designated genotype run in 3 replicates each. Left, standard wort conditions, right, wort supplemented with leucine.

crossed with a lager strain spore clone (lager spore a, Table 1) possessing the ability to ferment maltotriose. This feature was indeed passed down to the resulting hybrid as demonstrated by higher RDF values (Fig. 2D, Table S3). Overall, these results showed that strains containing specific point mutations can be identified and isolated with FIND-IT and the traits can subsequently be passed down to other strains in e.g. an ale/lager breeding program.

3.2. *IAH1* deletion provides only limited increase in isoamyl acetate production in the bioethanol strain

Esters are important flavor-active compounds contributing to the final beer taste and aroma [13]. Deletion of *IAH1* has been shown to positively influence the production of esters, particularly acetate esters such as isoamyl acetate or 2-phenylethyl acetate [51,52]. However, such a trait does not possess a phenotype that would be feasible to screen for. The presented technology is therefore beneficial for investigating the effect of the *IAH1* gene inactivation. The $IAH1^{G386A}$ mutation, finding a premature STOP codon in the coding sequence and thus the gene disruption was identified in the library 1 prepared as described above of the PE-2 spore (Fig. S2). In order to obtain the *iah1* phenotype in the POF^- background, further breeding with the PE-2 a fdc1 was performed. As a result, haploid spores with the *fdc1 iah1* genotype, and homozygous as well heterozygous diploid $IAH1^{G386A}$ mutants (Table 1) were obtained. All strains were fermented in wort and finished fermentations analyzed for concentration of selected flavor-active compounds. As expected, there was no significant difference in production of esters when strains with at least one functional allele of *IAH1* gene were compared. Surprisingly haploid *iah1* mutants seemed to produce slightly less isoamyl acetate and 2-phenylethyl acetate on average (Fig. 3A). However, it is necessary to note that different haploid spores differed in production of esters resulting in larger error bars when the numbers were averaged (Fig. S3). On the other hand, diploid *iah1* mutants produced more acetate esters (Fig. 3A). The results also showed slightly higher production of alcohol precursors (isoamyl alcohol and 2-phenylethanol) in diploid strains independently on the present mutation (Fig. 3B). The levels of other esters (except for isobutyl acetate that followed the trend described above) such as ethyl acetate or medium chain fatty acid (MCFA) derived esters did not show significant differences (Fig. S4). Similar trend was observed when all the strains were fermented in wort with supplementation of leucine General increase in production of isoamyl acetate (Fig. 3A) and isoamyl alcohol (Fig. 3B) was observed in all the strains. This was more pronounced in diploid *iah1* mutants where the concentration of the isoamyl acetate was twice as high when compared to the wild type strain. Moreover, lower concentrations of 2-phenylethyl acetate and 2-phenylethanol due to the leucine excess were apparent (Figs. 3A, 3B).

3.3. Missense mutations of *TYR1* gene identified in a lager yeast background change flavor profiles of the production strain

To introduce gene knockout mutations into genetically complex brewer's yeast such as lager strains may represent an issue and would require several rounds of mutagenesis treatments and mutant isolation. This could induce undesired genetic changes into already complicated genomes and result in an unpredictability of the resulting phenotype. We attempted to observe a possibility to identify gain of function mutations using our approach in a lager yeast strain (Table 1). Recently, several mutations in genes encoding enzymes involved in aromatic amino acid synthesis pathway were identified to be causative for the increased production of phenylalanine-derived alcohol and ester in wine yeast strains [53]. Three different mutations (G550A, G562A and C629T leading to amino acid substitutions) in *TYR1* gene, encoding a prephenate dehydrogenase involved in last steps of tyrosine biosynthesis were identified in a mutagenized library of the lager yeast strain (Fig. 4A). The resulting fractional abundance of the mutations in single colony isolates was around 25 %, suggesting that one allele of the gene contained the mutation in supposedly tetraploid background (Fig. 4A). Isolated strains were cultivated at standard lager conditions and the finished fermented wort analyzed. Surprisingly, while the Tyr1 A188T mutant did not show any significant difference in concentration of flavor active compounds when compared to the wild type strain, the other two mutations had a complex impact on the production of acetate esters and fusel alcohol (Fig. 4B, Fig. 4C). In contrast to previous findings there was a significant decrease in production of all acetate esters in case of Tyr1 A184T mutant. On the other hand, in case of Tyr1 T210I only a decrease of amino-acid derived esters could be observed while ethyl acetate concentration was higher when compared to the wild type strain (Fig. 4B). Interestingly, this was accompanied by lower levels of fusel alcohol precursors in Tyr1 T210I mutant, while only a very slight difference (except for isoamyl alcohol concentration in Tyr1 A184T mutant) was seen in case of the other two mutations (Fig. 4C). We did not observe any significant differences in the fermentation performance monitored by CO_2 accumulation and RDF measurement (Fig. S5) when compared the parental strain and the mutants. Further supplementation of the wort with phenylalanine resulted in a dramatic increase in production of 2-phenylethanol and 2-phenylethyl acetate in all strains. However, the general trend observed before in standard wort, i.e. lower level of amino acid derived flavor compounds in Tyr1 T210I and lower level of all acetate esters in Tyr1 A188T when compared to the lager strain and Tyr1 A188T was preserved (Figs. 4B, 4C).

3.4. Heterothallic *S. eubayanus* facilitates breeding and brings flavor diversity into novel lager yeast

An *S. eubayanus* strain (Table 1) isolated from forests in Patagonia [33] was sporulated and spores from several asci examined by the halo assay with mating pheromone hypersensitive *S. cerevisiae* strains (Table 1). No spore seemed to emit a mating pheromone and the strain thus obviously retained its homothallic nature (Fig. 5A). The strain was then subject to the mutagenesis protocol and the mutagenized population screened for the presence of a specific mutation in *HO*-like gene (C238T) that would allow for isolation of a heterothallic strain. The mutation causes an introduction of a premature STOP codon into the coding sequence and thus presumably a disruption of the gene. Even though such mutation was previously found in some heterothallic natural *S. cerevisiae* isolates [54], it has not been identified (neither another *HO* or non-*HO* mutation leading to heterothallism [55] in *S. eubayanus* to date. The mutant strain was subsequently isolated from the population via the enrichment of the mutant in the population up to single colony level. As expected, the fractional abundance of the mutation of the clonal population was at 50 % corresponding to the diploid nature of the strain suggesting that one allele of the gene was affected. After spore isolation the phenotype was observed via halo assay and isolated spores confirmed for 100 % presence of the mutation (Fig. 5A). Acquired HO^- spores were tested for growth in wort media in 96-well plates in anaerobic conditions at 16 °C. There was no significant growth difference apparent among spores from different asci pointing at quite homozygous nature of the yeast (Fig. S6). Selected spores were then crossed with a haploid *S. cerevisiae* strain (S.c. PE-2 a or α) and a lager yeast spore (lager spore a) to produce novel lager strains (novel lager 1, novel lager 2) via mass mating. Hybridization frequency was 20–26 % and 10–16 % for novel lager 1 and novel lager 2, respectively (Fig. 5B). In case of mating with pure *S. cerevisiae* spores when the cells plated after mating were grown at 37 °C (non-permissive temperature for *S. eubayanus*) essentially almost every second colony was a hybrid clone (40–49 %). As a control experiment haploid *S. cerevisiae* strains and the lager yeast spore were crossed with zymolyase treated asci of the wild type *S. eubayanus* strain. There hybridization frequency was significantly lower (3.2 % for *S. cerevisiae* x *S. eubayanus* and 1.8 % for lager spore x *S. eubayanus*) than for heterothallic *S. eubayanus* spores (Table S4). Selected novel hybrids as well as parental strains were

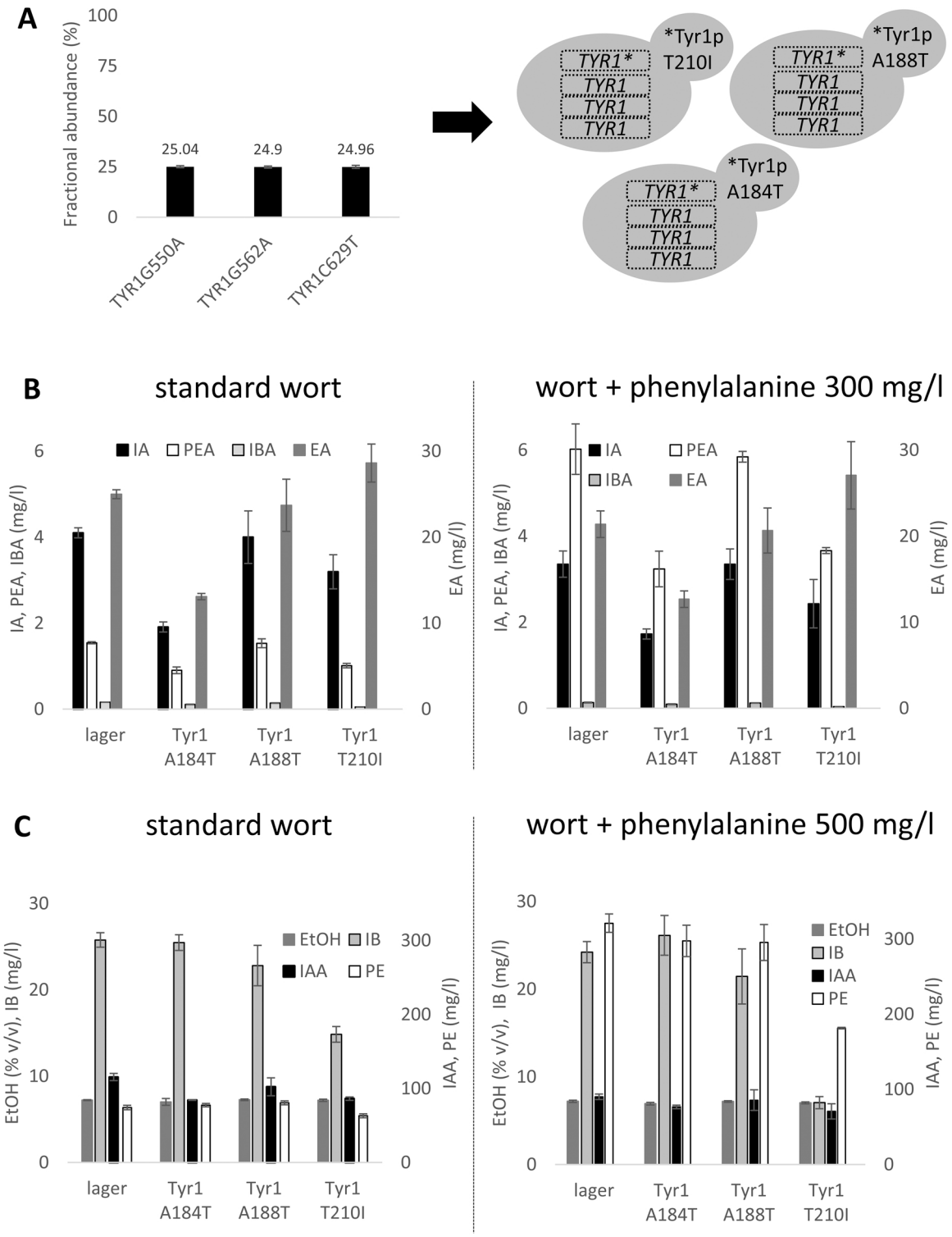

Fig. 4. Gain of function mutations in *TYR1* gene in a 4 n lager yeast. A) The graph shows fractional abundance of three mutations in *TYR1* gene ($TYR1^{G550A}$, $TYR1^{G562A}$ and $TYR1^{C629T}$) in single colonies of lager yeast strain. The values are average of 5 clones after restreak of a single colony isolated from the mutagenized library. Right, illustration of resulting mutants with altered Tyr1p (Tyr1p A184T, Tyr1p A188T, Tyr1p T210I). B) The graphs show concentration of isoamyl acetate (IA), 2-phenylethyl acetate (PEA), isobutyl acetate (IBA) and ethyl acetate (EA) in finished wort fermentations of lager yeast strain (lager) and its *TYR1* mutants. Ethyl acetate is displayed on secondary y axis. Left, standard wort conditions, right, wort supplemented with phenylalanine. The values are average of three independent replicates. Error bars represent standard deviation (N = 3). C) The graphs show concentration of isoamyl alcohol (IAA), 2-phenylethanol (PE), isobutanol (IB) and ethanol (EtOH) in finished wort fermentations of lager yeast strain (lager) and its *TYR1* mutants. Isoamyl alcohol and 2-phenylethanol is displayed on secondary y axis. Left, standard wort conditions, right, wort supplemented with phenylalanine. The values are average of three independent replicates. Error bars represent standard deviation (N = 3).

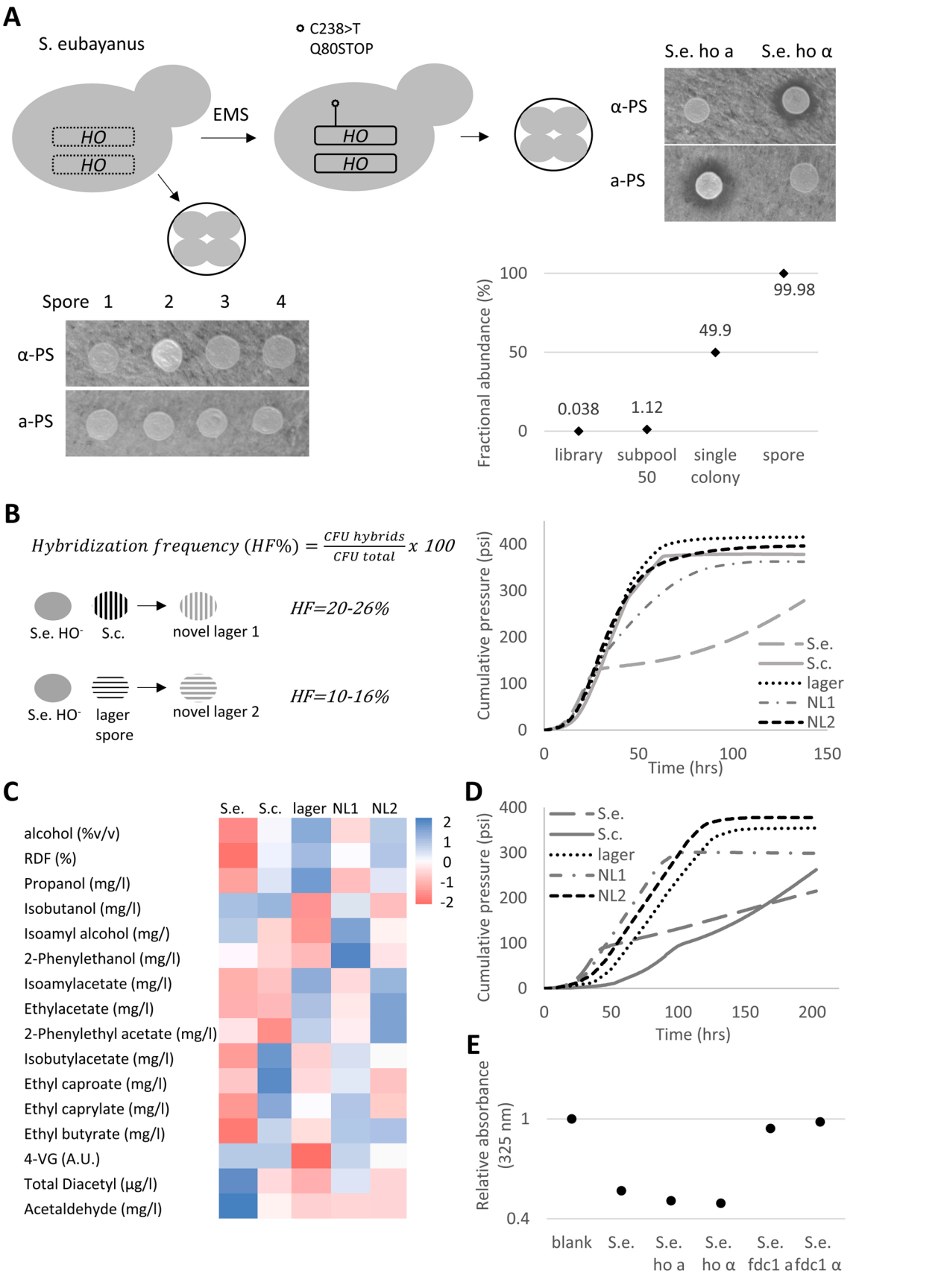

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Fig. 5. Isolation of haploid *S. eubayanus* strain and its breeding capabilities. A) Schematic illustration of the identification of C238T mutation in *HO*-like gene in the population of *S. eubayanus* strain and isolation of the subsequent mutant spores. Spores with HO^{C238T} mutation (S.e. ho a, S.e. ho α) along with wild type spores were examined for production of mating pheromones using pheromone sensitive tester strain (α-PS – α pheromone sensitive, a-PS – a pheromone sensitive). The graph of fractional abundance shows the frequency of the HO^{C238T} mutation in the population of the original library (~2500 cells), subpool 50 (~50 cells), single colony and spore clone. B) Illustration of breeding of heterothallic *S. eubayanus* haploid pure lines (S.e. HO^-) with selected PE-2 (*S.c.*) and lager yeast (lager spore) spores. Hybridization frequency calculated using the displayed formula is given for each example. The chart displays CO_2 accumulation curves expressed as cumulative pressure of *S. eubayanus* (S.e.), PE-2 (S.c.), lager yeast (lager) and their novel lager hybrids (NL1, NL2) fermenting wort at 16 °C. C) Flavor profiles of *S. eubayanus* (S.e.), PE-2 (S.c.), lager yeast (lager) and their novel lager hybrids (NL1, NL2). Color coding is based on a Z-score calculated from absolute values in the data set (absolute values are in Table S5. The color scale is displayed. D) The chart displays CO_2 accumulation curves expressed as cumulative pressure of *S. eubayanus* (S.e.), PE-2 (S.c.), lager yeast (lager) and their novel lager hybrids (NL1, NL2) fermenting wort at 10 °C. E) Chart displays a relative absorbance at 325 nm of fermented samples of *S. eubayanus* (S.e.), its haploid spores (S.e. ho a, S.e. ho α) and haploid $FDC1^{G477A}$ (S.e. fdc1 a, S.e. fdc1 α) mutants in YPD medium with 1 mM ferulic acid. The relative value was calculated as the average absorbance at 325 nm (N = 3) of with the particular strains divided by the value of non-inoculated sample of YPD with 1 mM ferulic acid (blank).

examined for fermentation properties and flavor production in wort at 16 °C. CO_2 production was monitored using the gas pressure monitoring system (Fig. 5B). Even though wild type *S. eubayanus* apparently did not ferment all the sugars in wort (and did not finish fermentation before the experiment was ended, Fig. 5B) reaching low RDF as well as alcohol levels, it produced substantially high production of fusel alcohols (Fig. 5C). Level of common esters was low and there was high concentration of common off-flavors such as 4-VG and diacetyl. Poor fermentation properties and ester formation could be compensated by a *S. cerevisiae* parent. Non-brewing *S. cerevisiae* partner (PE-2) brought the improved maltose uptake and so increased alcohol production and final RDF value as well as higher production of common esters. The level of acetate esters was even higher when compared to the *S. cerevisiae* strain and the hybrid kept high levels of aromatic alcohols and fatty acid-derived esters (a feature typical for the particular *S. cerevisiae* strain). Apparent absence of maltotriose fermentation was compensated by breeding with a lager spore increasing RDF (slightly lower than the optimized production strain though). Ester levels were higher as well as production of some alcohols when compared to the production lager yeast strain (Fig. 5C, Table S5). Interestingly, the novel lager hybrid strains outperformed the production lager strain in terms of fermentation speed at 10 °C, showing that the wild *S. eubayanus* can broaden the range of possible fermentation temperatures (Fig. 5D). While the production of diacetyl could also be lowered by breeding, 4-VG production remained high in the hybrids masking the other positive flavors. Heterothallic *S. eubayanus* strains were then subject to another round of mutagenesis and $FDC1^{G477A}$ mutants not converting ferulic acid (Fig. 5E) isolated using the described technology above. POF^- novel lager hybrids, novel lager 3 and novel lager 4 were obtained by breeding with the *S. cerevisiae* POF^- strain (S.c. PE-2 a fdc1) and the lager yeast spore, respectively. The resulting strains did not produce significant levels of 4-VG after fermentation in wort while keeping the production of other flavors at comparable levels to the POF^+ (NL1, NL2) hybrids (Fig. S7, Table S6).

4. Discussion

Emergence of mutations is a natural phenomenon that drives evolution and adaptation of species [56]. Conventional mutagenesis using various physical or chemical mutagens increases the mutation rate in the affected population and thus increases the chance of occurrence of a specific mutation in the population [42]. It has been used for decades to isolate mutants with new variants of genes [57]. However, absence of any trait specific selection method or high throughput phenotypic screening assays make it virtually impossible to screen thousands of cells for often non-selectable phenotypes such as flavor production modification in particularly complex industrial yeasts. However, as shown above the FIND-IT method [31], which uses the sensitivity of ddPCR [58] enables the detection of specific mutations in large strain-agnostic yeast libraries mutagenized using EMS. EMS treatment leads to guanine alkylation and subsequently GC to AT transitions [59] allowing for generation of both nonsense or missense mutations. The mutation rate and so the chance for finding a mutation of interest can be increased by multiple rounds of mutagenesis [60,61]. At the same time, a single strain library can be screened for several different mutations in a very short time. Even though there is some level of uncertainty in the individual library preparation, and it was not the purpose of the study to precisely monitor the mutation load in the prepared libraries, we generally conclude that two successive rounds of mutagenesis (in optimal strain-dependent treatment conditions) appear to be sufficient to detect the mutation of interest. For more quantitative evaluation of the mutation load in the population, approaches such as monitoring the presence of auxotrophic mutations and respiratory deficiency [60] or next generation sequencing [61] can be used when needed.

As a proof of concept we introduced and identified a nonsense mutation leading to the absence of 4-VG production in a bioethanol strain. Several other attempts to eliminate the undesirable off-flavor have been documented in non-domesticated strains, to apply such strains in a brewer's yeast breeding program. The approaches covered breeding [62], mutagenesis [63] and GM technologies [19]. Even though a phenotypic screening assay exists [35], throughput of the approach used here is significantly higher due to the pool of thousands of cells in the original library as also shown recently [31]. Biofuel production strains have a potential for brewing [64,65]. Such strains can be used in a wide variety of industrial conditions and temperatures. Our results also showed higher levels of MCFA-derived esters production and fast fermentation of simple sugars as a feature that can be inherited by their hybrids. Moreover, hybrids with an ale strain outperformed both parental strains in terms of fermentation speed pointing at hybrid vigor [66].

Once the library is established it can be used for finding more mutations in the population. A nonsense mutation of *IAH1* was found in the same library of the bioethanol strain as the mutation leading to the elimination of 4-VG production. It has been shown that *IAH1* deletion leads to an increase in formation of particularly acetate esters [51,52,67]. We observed similar but not dramatic effect only in the diploid strain with homozygous nonsense mutation. The difference we observed in haploid strains was rather insignificant and presumably driven by differences among various spore clones due to the documented high level of heterozygosity of the strain [68]. This could also imply potential differences in *ATF1* or *ATF2* encoding acetyltransferases as the importance of balance between acetyltransferase and esterase activity was shown previously [69]. There were also higher levels of alcohol precursors in diploids, thus resulting in the abundance of the corresponding esters in diploid mutants. This was even more pronounced when wort contained excess of leucine. This provides higher flux when the amino acid is degraded via the Ehrlich pathway resulting in higher concentrations of isoamyl alcohol and the corresponding ester [70]. Altogether as suggested before [51] complex regulation of aroma production might limit the effect of the esterase disruption. As mentioned above availability and excess of precursor amino acids [71] and/or potentially different variants of *ATF* genes [72] may have a larger impact on the final ester concentration.

Changes in production of amino acid-derived esters as a consequence

of catabolism of amino acids (particularly branched chain or aromatic amino acids) may be caused by an excess of an amino acid in cultivation medium [71] or increased flux through the anabolic pathways [73]. The latter may be driven by mutations causing the release of feedback inhibition of de novo synthesis of the amino acids. These phenotypes can be selected as the causative mutations often confer resistance to toxic analogues of amino acids [74,75]. Recently an effect of several mutations on production of 2-phenylethanol and 2-phenylethyl acetate, two floral aroma compounds [73] was investigated in wine yeast [53]. We isolated mutants with presumably the most potent mutations of *TYR1* [53] in the population of a polyploid lager yeast strain. In contrast to the previous findings, we observed that two of the mutations led to lower production of esters of flavor compounds in wort fermentations, i.e. the effect opposite to what was described before [53]. when the mutant strains It was suggested before that the flux towards phenyl pyruvate could be driven by lower activity of prephenate dehydrogenase [53] supported by the evidence that the mutants accumulated phenylalanine extracellularly and showed reduced intracellular tyrosine formation as shown also in another study [76]. However, based on our results we hypothesize that in the lager strain background the mutations rather lead to an increased activity of Tyr1. This could be the result of an increased flux towards 4-hydroxyphenylpyruvate. Whether the reason could be a disturbed regulation of the Ehrlich pathway or a redirection of enzymes towards degradation of tyrosine remains unknown without further investigation. The fact is that in case of Tyr1 T210I lower production of fusel alcohols resulted in a drop in production of esters whereas ethyl acetate (an ester not related to AA metabolic pathways) levels increased. On the other hand, Tyr1 A184T mutants showed low level of all esters not necessarily linked to significant decrease in the precursor alcohol level. We hypothesize that there could be a redox NADPH/$NADP^+$ imbalance caused by different demand of the modified enzyme for $NADP^+$ cofactor. Interestingly, the esterification reaction catalyzed by acetyltransferase enzymes also uses the same cofactors [77]. However, besides considering the use of a very different strain and conditions we cannot omit that heterozygosity of the mutation could also play a role as shown before [53]. Altogether it seems that one single base substitution in an enzyme of an amino acid synthetic pathway may lead to quite significant changes in flavor profiles of the mutant strains. However, despite it does not seem likely based on the observed phenotype where the strain fitness and performance were not affected, one cannot simply rule out a potential off-target effect of the mutagenesis procedure. For this further investigation such as whole genome sequencing of the resulting mutant strains would be needed.

Currently used production lager yeast strains, long time designated as *Saccharomyces pastorianus* are hybrids of *S. cerevisiae* and *S. eubayanus* [78]. Existence of the hybrids over the decades in the specific industrial settings resulted in limited genotypic and thus phenotypic (particularly flavor and aroma) diversity [79]. In addition, some interesting features may have been lost over time. Breeding of different strains and thus combining different traits was shown to be a way to bring more diversity into the production strain portfolio [80]. However, the alloploid nature and multiple hybridization events of the species resulted in genomic changes (chromosomal rearrangements and aneuploidy) leading to low sporulation capabilities and/or limited spore viability [81]. This makes it difficult to combine new traits within currently existing domesticated strains and select for new phenotypes in a predictable manner even though such attempts have also been documented [82]. In recent years interspecies hybridizations with non-*cerevisiae* strains isolated from the wild have been attempted to improve the phenotypic diversity [80,83,84]. The standard hybrid generation methods used for creation of novel hybrids rely on rather rare events such as loss of heterozygosity at mating type locus (so called rare mating) and require some kind of selection [85] or spore-to-spore mating techniques using a micromanipulator [86]. Mass mating technique [55,84] requires selection to increase the chance of the hybrid appearance and uses a preceding step of sporulation and so meiotic recombination. It increases the uncertainty of the resulting hybrid phenotype and also the risk of losing or altering the desired traits. The development of a heterothallic *S. eubayanus* strain described here overcomes these challenges as it does not require any selection for hybrids. Heterothallism is well known and described in *S. cerevisiae* [87] and the role of HO endonuclease elucidated [88]. It was shown that heterologous expression of *S. cerevisiae HO* in *S. eubayanus* led to forced mating type switching and formation of allotetraploid hybrids [89]. Recently, deletion of *HO* in S. eubayanus using CRISPR-Cas9 genome editing led to generation of stable haploid lineages for research purposes [90]. Our results document that a nonsense mutation in *SeHO* causes inability of mating type switching in *S. eubayanus* and thus enables to keep pure haploid lines that may be used for generation of commercial de novo hybrids. Frequency of hybridization with *S. cerevisiae* partners is higher than reported in some previous studies [84,86] and sufficient to avoid requirements for selection or laborious high throughput screenings. Generated novel hybrids broaden the range of fermentation temperature similarly to previous studies [55,84,85] when they outperform the parental strains at 10 °C. In addition the flavor profiles change as *S. eubayanus* serves as a rich source of fusel alcohols (as also described previously in [91]) or volatile thiols [92], while *S. cerevisiae* counterpart provides the capacity to produce esters. In general the novel hybrids produce higher concentration of especially acetate esters than their respective parents as mentioned in some previous studies [84,85]. The development of *S. eubayanus* pure haploid lines not only greatly facilitates breeding and creation of diverse hybrids but provides a simple platform for potential tailor-made changes in the genome. As the wild strain produces 4-VG as an off-flavor, a feature inherited by the novel hybrids, its elimination using the method described herein in heterothallic haploids serves as a proof. All this without the need for rather complex breeding programs [62,93], semi high throughput phenotypic screening [63] or advanced GMO tools [19,90].

In summary, we show that the FIND-IT method can be used for identification and subsequent isolation of nonsense or missense mutations in wide variety of yeast strains from a bioethanol strain, over an ale strains to lager hybrid strains and/or wild isolates. We demonstrate i) reduced production of off-flavors (such as 4-VG) of the mutant strain in brewing settings and/or breeding programs, ii) modified production of other flavor-active compounds by the isolation of mutants with disrupted genes or genes with modified sequence leading to amino acid substitutions. Furthermore, isolation of the heterothallic *S. eubayanus* enables efficient breeding and creation of novel lager hybrids bringing more phenotypic options into the lager yeast portfolio. Even though the technique presented here has (as any other technology) its drawbacks (e.g. influence of potential off-target mutations on strain phenotype and fitness, repeated mutagenesis and screening rounds to obtain gene knock-outs in polyploid strains), and it was not the ambition of this study to compare it with advanced molecular biology tools, it is a non-GM technique. Therefore the isolated strains can be used in the food industry straightaway. As it can be seen the technology can be used for reverse engineering where GM tools cannot be applied and stresses out the importance of further research in the field of yeast flavor production for identification of causative mutations. It would increase the advantage of the method for improvement of very complex phenotypes such as flavor production where limited phenotypic selections exist, introduction of novel features into the current production strains and lead to faster generation of yeasts with tailor-made changes in their genomes and enhanced brewing related properties.

CRediT authorship contribution statement

Jochen Förster: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Vratislav Stovicek:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Toni Wendt:** Validation, Investigation, Conceptualization. **Michael Katz:** Writing – review

& editing, Writing – original draft, Supervision, Resources, Investigation. **Klaus Lengeler:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Magnus Rasmussen:** Validation, Formal analysis.

Declaration of Competing Interest

VS and JF are inventors of a patent application EP4029935A1, filed 14. 01. 2021 and published 20. 07. 2022 related to parts of this study. All authors were employed by Carlsberg A/S at the time of the research. Toni Wendt is a founder of Traitomic. The methodology presented in the manuscript is applied commercially by Traitomic A/S (www.traitomic.com), part of Carlsberg Group.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2024.05.006.

References

[1] Parapouli M, Vasileiadis A, Afendra A-S, Hatziloukas E. Saccharomyces cerevisiae and its industrial applications. AIMS Microbiol 2020;6(1):31. https://doi.org/10.3934/microbiol.2020001.
[2] Raihofer L, Zarnow M, Gastl M, Hutzler M. A short history of beer brewing. EMBO Rep 2022;23:e56355. https://doi.org/10.15252/embr.202256355.
[3] Mohd Azhar SH, Abdulla R, Jambo SA, Marbawi H, Gansau JA, Mohd Faik AA, et al. Yeasts in sustainable bioethanol production: a review. Biochem Biophys Rep 2017;10:52–61. https://doi.org/10.1016/j.bbrep.2017.03.003.
[4] Borodina I, Nielsen J. Advances in metabolic engineering of yeast Saccharomyces cerevisiae for production of chemicals. Biotechnol J 2014;9:609–20. https://doi.org/10.1002/biot.201300445.
[5] Wang S, Zhao F, Yang M, Lin Y, Han S. Metabolic engineering of Saccharomyces cerevisiae for the synthesis of valuable chemicals. Crit Rev Biotechnol 2023;0:1–28. https://doi.org/10.1080/07388551.2022.2153008.
[6] Kavšček M, Stražar M, Curk T, Natter K, Petrovič U. Yeast as a cell factory: current state and perspectives. Microb Cell Factor 2015;14:94. https://doi.org/10.1186/s12934-015-0281-x.
[7] Stovicek V, Holkenbrink C, Borodina I. CRISPR/Cas system for yeast genome engineering: advances and applications. FEMS Yeast Res 2017;17. https://doi.org/10.1093/femsyr/fox030.
[8] Nielsen J. Yeast systems biology: model organism and cell factory. Biotechnol J 2019;14:1800421. https://doi.org/10.1002/biot.201800421.
[9] Romero-Suarez D, Keasling JD, Jensen MK. Supplying plant natural products by yeast cell factories. Curr Opin Green Sustain Chem 2022;33:100567. https://doi.org/10.1016/j.cogsc.2021.100567.
[10] Bonatto D. The diversity of commercially available ale and lager yeast strains and the impact of brewer's preferential yeast choice on the fermentative beer profiles. Food Res Int 2021;141:110125. https://doi.org/10.1016/j.foodres.2021.110125.
[11] Gallone B, Steensels J, Prahl T, Soriaga L, Saels V, Herrera-Malaver B, et al. Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts. Cell 2016;166:1397–1410.e16. https://doi.org/10.1016/j.cell.2016.08.020.
[12] Lengeler KB, Stovicek V, Fennessy RT, Katz M, Förster J. Never change a brewing yeast? Why not, there are plenty to choose from. Front Genet 2020;11. https://doi.org/10.3389/fgene.2020.582789.
[13] Pires EJ, Teixeira JA, Brányik T, Vicente AA. Yeast: the soul of beer's aroma–a review of flavour-active esters and higher alcohols produced by the brewing yeast. Appl Microbiol Biotechnol 2014;98:1937–49. https://doi.org/10.1007/s00253-013-5470-0.
[14] Larroque MN, Carrau F, Fariña L, Boido E, Dellacassa E, Medina K. Effect of Saccharomyces and non-Saccharomyces native yeasts on beer aroma compounds. Int J Food Microbiol 2021;337:108953. https://doi.org/10.1016/j.ijfoodmicro.2020.108953.
[15] Gibson B, Geertman J-MA, Hittinger CT, Krogerus K, Libkind D, Louis EJ, et al. New yeasts—new brews: modern approaches to brewing yeast design and development. FEMS Yeast Res 2017;17. https://doi.org/10.1093/femsyr/fox038.
[16] Astola A, Durán-Guerrero E, Díaz AB, Lasanta C, Castro R. Impact of the genetic improvement of fermenting yeasts on the organoleptic properties of beer. Eur Food Res Technol 2023;249:1677–87. https://doi.org/10.1007/s00217-023-04251-8.
[17] Spencer JFT, Spencer DM. Genetic improvement of industrial yeasts. Annu Rev Microbiol 1983;37:121–42. https://doi.org/10.1146/annurev.mi.37.100183.001005.
[18] Stovicek V, Borodina I, Forster J. CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains. Metab Eng Commun 2015;2:13–22. https://doi.org/10.1016/j.meteno.2015.03.001.
[19] Mertens S, Gallone B, Steensels J, Herrera-Malaver B, Cortebeek J, Nolmans R, et al. Reducing phenolic off-flavors through CRISPR-based gene editing of the FDC1 gene in Saccharomyces cerevisiae x Saccharomyces eubayanus hybrid lager beer yeasts. PLoS One 2019;14. https://doi.org/10.1371/journal.pone.0209124.
[20] Denby CM, Li RA, Vu VT, Costello Z, Lin W, Chan LJG, et al. Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer. Nat Commun 2018;9:965. https://doi.org/10.1038/s41467-018-03293-x.
[21] Molitor RW, Roop JI, Denby CM, Depew CJ, Liu DS, Stadulis SE, et al. The sensorial and chemical changes in beer brewed with yeast genetically modified to release polyfunctional thiols from malt and hops. Fermentation 2022;8:370. https://doi.org/10.3390/fermentation8080370.
[22] Eriksson D, Custers R, Edvardsson Björnberg K, Hansson SO, Purnhagen K, Qaim M, et al. Options to Reform the European Union Legislation on GMOs: risk governance. Trends Biotechnol 2020;38:349–51. https://doi.org/10.1016/j.tibtech.2019.12.016.
[23] Ishii T, Araki M. Consumer acceptance of food crops developed by genome editing. Plant Cell Rep 2016;35:1507–18. https://doi.org/10.1007/s00299-016-1974-2.
[24] Wesseler J, Kleter G, Meulenbroek M, Purnhagen KPEU. regulation of genetically modified microorganisms in light of new policy developments: Possible implications for EU bioeconomy investments. Appl Econ Perspect Policy 2023;45:839–59. https://doi.org/10.1002/aepp.13259.
[25] Duewer DL, Kline MC, Romsos EL, Toman B. Evaluating droplet digital PCR for the quantification of human genomic DNA: converting copies per nanoliter to nanograms nuclear DNA per microliter. Anal Bioanal Chem 2018;410:2879–87. https://doi.org/10.1007/s00216-018-0982-1.
[26] Quan P-L, Sauzade M, Brouzes E. dPCR: a technology review. Sensors 2018;18. https://doi.org/10.3390/s18041271.
[27] Bell AD, Usher CL, McCarroll SA. Analyzing Copy Number Variation with Droplet Digital PCR. In: Karlin-Neumann G, Bizouarn F, editors. Digital PCR: Methods and Protocols. New York, NY: Springer; 2018. p. 143–60. https://doi.org/10.1007/978-1-4939-7778-9_9.
[28] Kamitaki N, Usher CL, McCarroll SA. Using Droplet Digital PCR to Analyze Allele-Specific RNA Expression. In: Karlin-Neumann G, Bizouarn F, editors. Digital PCR: Methods and Protocols. New York, NY: Springer; 2018. p. 401–22. https://doi.org/10.1007/978-1-4939-7778-9_23.
[29] Kuypers J, Jerome KR. Applications of digital PCR for clinical microbiology. J Clin Microbiol 2017;55:1621–8. https://doi.org/10.1128/JCM.00211-17.
[30] Olmedillas-López S, García-Arranz M, García-Olmo D. Current and emerging applications of droplet digital PCR in oncology. Mol Diagn Ther 2017;21:493–510. https://doi.org/10.1007/s40291-017-0278-8.
[31] Knudsen S, Wendt T, Dockter C, Thomsen HC, Rasmussen M, Egevang Jørgensen M, et al. FIND-IT: Accelerated trait development for a green evolution. Sci Adv 2022;8:eabq2266. https://doi.org/10.1126/sciadv.abq2266.
[32] Skadhauge B., Knudsen S., Hambraeus G., Wendt T., Rasmussen M., Østerberg J.T., et al. Methods for preparing mutant plants or microorganisms. US20220312707A1, 2022.
[33] Libkind D, Hittinger CT, Valério E, Gonçalves C, Dover J, Johnston M, et al. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. PNAS 2011;108:14539–44. https://doi.org/10.1073/pnas.1105430108.
[34] Wasmer M. Roads forward for European GMO policy—uncertainties in wake of ECJ judgment have to be mitigated by regulatory reform. Front Bioeng Biotechnol 2019;7. https://doi.org/10.3389/fbioe.2019.00132.
[35] Mertens S, Steensels J, Gallone B, Souffriau B, Malcorps P, Verstrepen KJ. Rapid screening method for phenolic off-flavor (POF) Production in Yeast. J Am Soc Brew Chem 2017;75:318–23. https://doi.org/10.1094/ASBCJ-2017-4142-01.
[36] Colomer MS, Chailyan A, Fennessy RT, Olsson KF, Johnsen L, Solodovnikova N, et al. Assessing population diversity of *Brettanomyces* yeast species and identification of strains for brewing applications. Front Microbiol 2020;11. https://doi.org/10.3389/fmicb.2020.00637.
[37] Kempf C, Lengeler K, Wendland J. Differential stress response of Saccharomyces hybrids revealed by monitoring Hsp104 aggregation and disaggregation. Microbiol Res 2017;200:53–63. https://doi.org/10.1016/j.micres.2017.03.009.
[38] Treco DA, Winston F. Growth and manipulation of yeast. 13.2.1-13.2.12 Curr Protoc Mol Biol 2008;82. https://doi.org/10.1002/0471142727.mb1302s82.
[39] Sumner-Smith M, Bozzato RP, Skipper N, Davies RW, Hopper JE. Analysis of the inducible MEL1 gene of Saccharomyces carlsbergensis and its secreted product, alpha-galactosidase (melibiase). Gene 1985;36:333–40. https://doi.org/10.1016/0378-1119(85)90188-x.
[40] Ambrona J, Maqueda M, Zamora E, Ramírez M. Sulfometuron resistance as a genetic marker for yeast populations in wine fermentations. J Agric Food Chem 2005;53:7438–43. https://doi.org/10.1021/jf0514468.
[41] Gjermansen C, Nilsson-Tillgren T, Petersen JGL, Kielland-Brandt MC, Sigsgaard P, Holmberg S. Towards diacetyl-less brewers' yeast. Influence of ilv2 and ilv5 mutations. J Basic Microbiol 1988;28:175–83. https://doi.org/10.1002/jobm.3620280304.
[42] Winston F. EMS and UV mutagenesis in yeast. Chapter 13:Unit 13.3B Curr Protoc Mol Biol 2008. https://doi.org/10.1002/0471142727.mb1303bs82.

[43] Wendt T., Olsen O., Knudsen S., Thomsen H.C., Skadhauge B., Rasmussen M.W., et al. Method to Screen For a Mutant Within a Population of Organisms By Applying a Pooling and Splitting Approach. US20190194723A1, 2019.
[44] Olaniran AO, Hiralal L, Mokoena MP, Pillay B. Flavour-active volatile compounds in beer: production, regulation and control. J Inst Brew 2017;123:13–23. https://doi.org/10.1002/jib.389.
[45] Trachman H, Saletan LT. Simplified determination of beer volatiles by gas chromatography. Proc Annu Meet - Am Soc Brew Chem 1969;27:19–28. https://doi.org/10.1080/00960845.1969.12007094.
[46] Argueso JL, Carazzolle MF, Mieczkowski PA, Duarte FM, Netto OVC, Missawa SK, et al. Genome structure of a Saccharomyces cerevisiae strain widely used in bioethanol production. Genome Res 2009;19:2258–70. https://doi.org/10.1101/gr.091777.109.
[47] Basso LC, Amorim HVD, Oliveira AJD, Lopes ML. Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res 2008;8:1155–63. https://doi.org/10.1111/j.1567-1364.2008.00428.x.
[48] Coghe S, Benoot K, Delvaux F, Vanderhaegen B, Delvaux FR. Ferulic acid release and 4-vinylguaiacol formation during brewing and fermentation: indications for feruloyl esterase activity in Saccharomyces cerevisiae. J Agric Food Chem 2004;52:602–8. https://doi.org/10.1021/jf0346556.
[49] Mukai N, Masaki K, Fujii T, Kawamukai M, Iefuji H. PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in Saccharomyces cerevisiae. J Biosci Bioeng 2010;109:564–9. https://doi.org/10.1016/j.jbiosc.2009.11.011.
[50] Fay JC, Liu P, Ong GT, Dunham MJ, Cromie GA, Jeffery EW, et al. A polyploid admixed origin of beer yeasts derived from European and Asian wine populations. PLOS Biol 2019;17:e3000147. https://doi.org/10.1371/journal.pbio.3000147.
[51] Dank A, Smid EJ, Notebaart RA. CRISPR-Cas genome engineering of esterase activity in Saccharomyces cerevisiae steers aroma formation. BMC Res Notes 2018;11:682. https://doi.org/10.1186/s13104-018-3788-5.
[52] Fukuda K, Yamamoto N, Kiyokawa Y, Yanagiuchi T, Wakai Y, Kitamoto K, et al. Brewing properties of sake yeast whose EST2 gene encoding isoamyl acetate-hydrolyzing esterase was disrupted. J Ferment Bioeng 1998;85:101–6. https://doi.org/10.1016/S0922-338X(97)80362-5.
[53] Cordente AG, Solomon M, Schulkin A, Leigh Francis I, Barker A, Borneman AR, et al. Novel wine yeast with ARO4 and TYR1 mutations that overproduce "floral" aroma compounds 2-phenylethanol and 2-phenylethyl acetate. Appl Microbiol Biotechnol 2018;102:5977–88. https://doi.org/10.1007/s00253-018-9054-x.
[54] Ezov TK, Chang S-L, Frenkel Z, Segrè AV, Bahalul M, MURRAY AW, et al. Heterothallism in Saccharomyces cerevisiae isolates from nature: effect of HO locus on the mode of reproduction. Mol Ecol 2010;19:121–31. https://doi.org/10.1111/j.1365-294X.2009.04436.x.
[55] Hebly M, Brickwedde A, Bolat I, Driessen MRM, de Hulster EAF, van den Broek M, et al. S. cerevisiae × S. eubayanus interspecific hybrid, the best of both worlds and beyond. FEMS Yeast Res 2015;15. https://doi.org/10.1093/femsyr/fov005.
[56] Lang GI, Murray AW. Estimating the per-base-pair mutation rate in the yeast Saccharomyces cerevisiae. Genetics 2008;178:67–82. https://doi.org/10.1534/genetics.107.071506.
[57] Chambers PJ, Bellon JR, Schmidt SA, Varela C, Pretorius IS. Non-Genetic Engineering Approaches for Isolating and Generating Novel Yeasts for Industrial Applications. In: Satyanarayana T, Kunze G, editors. Yeast Biotechnology: Diversity and Applications. Dordrecht: Springer Netherlands; 2009. p. 433–57. https://doi.org/10.1007/978-1-4020-8292-4_20.
[58] Parkin B. Rare variant quantitation using droplet digital PCR. Methods Mol Biol 2019;1881:239–51. https://doi.org/10.1007/978-1-4939-8876-1_18.
[59] Lee GS, Blonsky KS, Van On DL, Savage EA, Morgan AR, von Borstel RC. Base alterations in yeast induced by alkylating agents with differing Swain-Scott substrate constants. J Mol Biol 1992;223:617–26. https://doi.org/10.1016/0022-2836(92)90978-s.
[60] Abt TD, Souffriau B, Foulquié-Moreno MR, Duitama J, Thevelein JM. Genomic saturation mutagenesis and polygenic analysis identify novel yeast genes affecting ethyl acetate production, a non-selectable polygenic trait. Micro Cell 2016;3:159–75. https://doi.org/10.15698/mic2016.04.491.
[61] Lada AG, Stepchenkova EI, Waisertreiger ISR, Noskov VN, Dhar A, Eudy JD, et al. Genome-wide mutation avalanches induced in diploid yeast cells by a base analog or an APOBEC deaminase. PLoS Genet 2013;9:e1003736. https://doi.org/10.1371/journal.pgen.1003736.
[62] Krogerus K, Seppänen-Laakso T, Castillo S, Gibson B. Inheritance of brewing-relevant phenotypes in constructed *Saccharomyces cerevisiae* × *Saccharomyces eubayanus* hybrids. Micro Cell Fact 2017;16:66. https://doi.org/10.1186/s12934-017-0679-8.
[63] Diderich JA, Weening SM, van den Broek M, Pronk JT, Daran J-MG. Selection of Pof-Saccharomyces eubayanus variants for the construction of S. cerevisiae × S. eubayanus hybrids with reduced 4-vinyl guaiacol formation. Front Microbiol 2018;9. https://doi.org/10.3389/fmicb.2018.01640.
[64] Araújo TM, Souza MT, Diniz RHS, Yamakawa CK, Soares LB, Lenczak JL, et al. Cachaça yeast strains: alternative starters to produce beer and bioethanol. Antonie Van Leeuwenhoek 2018;111:1749–66. https://doi.org/10.1007/s10482-018-1063-3.
[65] Christofoleti-Furlan RM, Portugal CB, Varize CS, Muynarsk ESM, Alcarde AR, Basso LC. Unraveling Brazilian bioethanol yeasts as novel starters for high-gravity brewing. Food Res Int 2020;135:109282. https://doi.org/10.1016/j.foodres.2020.109282.
[66] Bernardes JP, Stelkens RB, Greig D. Heterosis in hybrids within and between yeast species. J Evol Biol 2017;30:538–48. https://doi.org/10.1111/jeb.13023.
[67] Fukuda K, Kiyokawa Y, Yanagiuchi T, Wakai Y, Kitamoto K, Inoue Y, et al. Purification and characterization of isoamyl acetate-hydrolyzing esterase encoded by the IAH1 gene of Saccharomyces cerevisiae from a recombinant Escherichia coli. Appl Microbiol Biotechnol 2000;53:596–600. https://doi.org/10.1007/s002530051662.
[68] Rácz HV, Mukhtar F, Imre A, Rádai Z, Gombert AK, Rátonyi T, et al. How to characterize a strain? The neglected influence of clonal heterogeneity on the phenotypes of industrial Saccharomyces. 07.19.211094. bioRxiv 2020;2020. https://doi.org/10.1101/2020.07.19.211094.
[69] Fukuda K, Yamamoto N, Kiyokawa Y, Yanagiuchi T, Wakai Y, Kitamoto K, et al. Balance of activities of alcohol acetyltransferase and esterase in Saccharomyces cerevisiae is important for production of isoamyl acetate. Appl Environ Microbiol 1998;64:4076–8.
[70] Engan S. Wort composition and beer flavour. I. the influence of some amino acids on the formation of higher aliphatic alcohols and esters. J Inst Brew 1970;76:254–61. https://doi.org/10.1002/j.2050-0416.1970.tb03292.x.
[71] He Y, Dong J, Yin H, Zhao Y, Chen R, Wan X, et al. Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer – a review. J Inst Brew 2014;120:157–63. https://doi.org/10.1002/jib.145.
[72] Verstrepen KJ, Van Laere SDM, Vanderhaegen BMP, Derdelinckx G, Dufour J-P, Pretorius IS, et al. Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. Appl Environ Microbiol 2003;69:5228–37. https://doi.org/10.1128/AEM.69.9.5228-5237.2003.
[73] Holt S, Mukherjee V, Lievens B, Verstrepen KJ, Thevelein JM. Bioflavoring by non-conventional yeasts in sequential beer fermentations. Food Microbiol 2018;72:55–66. https://doi.org/10.1016/j.fm.2017.11.008.
[74] Casalone E, Fia G, Barberio C, Cavalieri D, Turbanti L, Polsinelli M. Genetic and biochemical characterization of Saccharomyces cerevisiae mutants resistant to trifluoroleucine. Res Microbiol 1997;148:613–23. https://doi.org/10.1016/S0923-2508(97)88085-0.
[75] Fukuda K, Muromachi A, Watanabe M, Asano K, Takasawa S. Mutants producing high concentrations of the flavor components active amylalcohol and normal propanol in Saccharomyces cerevisiae. J Ferment Bioeng 1993;75:288–92. https://doi.org/10.1016/0922-338X(93)90153-Y.
[76] Fukuda K, Watanabe M, Asano K, Ouchi K, Takasawa S. Isolation and genetic study of p-fluoro-DL-phenylalanine-resistant mutants overproducing beta-phenethyl-alcohol in Saccharomyces cerevisiae. Curr Genet 1991;20:449–52. https://doi.org/10.1007/BF00334770.
[77] Hazelwood LA, Daran J-M, Maris AJA, van, Pronk JT, Dickinson JR. The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism. Appl Environ Microbiol 2008;74:2259–66. https://doi.org/10.1128/AEM.02625-07.
[78] Gibson B, Liti G. Saccharomyces pastorianus: genomic insights inspiring innovation for industry. Yeast 2015;32:17–27. https://doi.org/10.1002/yea.3033.
[79] Okuno M, Kajitani R, Ryusui R, Morimoto H, Kodama Y, Itoh T. Next-generation sequencing analysis of lager brewing yeast strains reveals the evolutionary history of interspecies hybridization. DNA Res 2016;23:67–80. https://doi.org/10.1093/dnares/dsv037.
[80] Steensels J, Snoek T, Meersman E, Picca Nicolino M, Voordeckers K, Verstrepen KJ. Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol Rev 2014;38:947–95. https://doi.org/10.1111/1574-6976.12073.
[81] Gorter de Vries AR, Pronk JT, Daran J-MG. Lager-brewing yeasts in the era of modern genetics. FEMS Yeast Res 2019;19. https://doi.org/10.1093/femsyr/foz063.
[82] Sanchez RG, Solodovnikova N, Wendland J. Breeding of lager yeast with Saccharomyces cerevisiae improves stress resistance and fermentation performance. Yeast 2012;29:343–55. https://doi.org/10.1002/yea.2914.
[83] Bellon JR, Eglinton JM, Siebert TE, Pollnitz AP, Rose L, de Barros Lopes M, et al. Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines. Appl Microbiol Biotechnol 2011;91:603–12. https://doi.org/10.1007/s00253-011-3294-3.
[84] Mertens S, Steensels J, Saels V, De Rouck G, Aerts G, Verstrepen KJ. A large set of newly created interspecific *Saccharomyces* hybrids increases aromatic diversity in lager beers. Appl Environ Microbiol 2015;81:8202–14. https://doi.org/10.1128/AEM.02464-15.
[85] Krogerus K, Magalhães F, Vidgren V, Gibson B. New lager yeast strains generated by interspecific hybridization. J Ind Microbiol Biotechnol 2015;42:769–78. https://doi.org/10.1007/s10295-015-1597-6.
[86] Krogerus K, Arvas M, De Chiara M, Magalhães F, Mattinen L, Oja M, et al. Ploidy influences the functional attributes of de novo lager yeast hybrids. Appl Microbiol Biotechnol 2016;100:7203–22. https://doi.org/10.1007/s00253-016-7588-3.
[87] Herskowitz I. Life cycle of the budding yeast Saccharomyces cerevisiae. Microbiol Rev 1988;52:536–53.
[88] Winge Ö., Roberts C. A Gene for Diploidization in Yeasts. Hagerup in Komm.; 1949.
[89] Alexander WG, Peris D, Pfannenstiel BT, Opulente DA, Kuang M, Hittinger CT. Efficient engineering of marker-free synthetic allotetraploids of Saccharomyces. Fungal Genet Biol 2016;89:10–7. https://doi.org/10.1016/j.fgb.2015.11.002.
[90] Molinet J, Urbina K, Villegas C, Abarca V, Oporto CI, Villarreal P, et al. A Saccharomyces eubayanus haploid resource for research studies. Sci Rep 2022;12:5976. https://doi.org/10.1038/s41598-022-10048-8.
[91] Gibson BR, Storgårds E, Krogerus K, Vidgren V. Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the

parental species Saccharomyces eubayanus. Yeast 2013;30:255–66. https://doi.org/10.1002/yea.2960.

[92] Krogerus K, Rettberg N, Gibson B. Increased volatile thiol release during beer fermentation using constructed interspecies yeast hybrids. Eur Food Res Technol 2023;249:55–69. https://doi.org/10.1007/s00217-022-04132-6.

[93] Turgeon Z, Sierocinski T, Brimacombe CA, Jin Y, Goldhawke B, Swanson JM, et al. Industrially applicable De Novo lager yeast hybrids with a unique genomic architecture: creation and characterization. Appl Environ Microbiol 2021;87:e02434-20. https://doi.org/10.1128/AEM.02434-20.