EXPLORING INTERSPECIFIC YEAST BREEDING FOR INDUSTRIAL APPLICATION

INTRODUCING AROMATIC DIVERSITY IN LAGER BEER

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Preface - Voorwoord

Try getting a PhD they said……..it will be fun, they said…… And true they were!

Hier ben ik dan, na legendarische uitspraken als “ik loop nog liever een ganse dag achter een vuilniskar dan mijn studies als bio-ingenieur verder te zetten” (december 2008, eerste examen periode aan de universiteit) en “Een doctoraat? Neen dank u, zo gek krijg je me nooit!” (juni 2013, net na het indienen van mijn masterthesis). Heb ik spijt dat ik toch de uitdaging aangegaan ben? Zeker en vast niet! De voorbije vijf jaar zijn voorbij gevlogen, met enkele laagtes maar vooral toch veel hoogtes en met als apotheose dit 140 pagina’s lange eindwerk. Het enige wat me nu nog rest is het bedanken van een aantal zeer belangrijke mensen.

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Rahel, Rita, Robbe, Ruben (Ruby), Sofie, Stijn (Spaepy), Supinya, Tim, Veerle, Victor, Yannick (Yannina) and Yudi. It has been an honour working together with you! Many thanks for the great time we had in and outside of the lab! Without such great colleagues, this thesis would not have been what it is today! Special thanks to my master thesis mentor Tim. I really enjoyed working together with you and it is safe to say that I owe a lot of my lab-skills, as well as part of my passion for science and yeast to you. To Jan and Brigida, for all the guidance, help and friendship during my PhD. To my past and current office buddies, thanks for the good time, the politically incorrect jokes and the interesting discussions we had -and still have! To Jeroen and Robbe, proud members of the awesome brewing crew. To Miguel, who introduced me into home brewing, showed me how to grill a whole lamb on a wood fire, who was always in for a (quick) beer after work and who I now consider as one of my best friends.

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Samenvatting

Bier is de gefermenteerde drank bij uitstek en is op wereldgebied - met een jaarlijkse productie van meer dan 1,96 miljard hectoliter - de derde meest geconsumeerde drank (na water en thee). Traditioneel bestaan er twee soorten bier, namelijk bieren van hoge ('ale')- en lage ('lager') gisting (meestal pilsbieren).

Pilsbieren, die 90% van de wereldwijde bierproductie vertegenwoordigen, worden exclusief gebrouwen met de pilsbiergist *Saccharomyces pastorianus*. Deze gist is geen pure gistsoort, maar is het resultaat van een interspecifieke kruising tussen enerzijds de alom bekende brouwers- en bakkersgist *Saccharomyces cerevisiae* en anderzijds een koude-tolerante en wilde gist *Saccharomyces eubayanus*. De beperkte genetische diversiteit binnen pilsbiergisten reflecteert zich in de geringe invloed van deze gisten op de smaak en het aroma van pilsbieren, zeker wanneer men dit vergelijkt met de overweldigende diversiteit aan bovengistingsbieren.

Hoewel het succes van pilsbieren grotendeels te danken is aan de typerende frisse smaak en hoge drinkbaarheid, wordt differentiatie en diversiteit steeds belangrijker. Dit om tegemoet te komen aan de dalende consumptie van pilsbieren, en om meet te gaan in de verandering van een meer globale biermarkt naar een niche-gedreven markt. De ontwikkeling van nieuwe pilsbiergisten kan helpen om een nieuw type bier te ontwikkelen, dat de verbinding maakt tussen enerzijds de diverse en aromatische bovengistingsbieren en anderzijds de frisse en vlot drinkende pilsbieren.

Het eerste hoofdstuk van deze thesis omvat een gedetailleerde literatuurstudie over de rol van gisten binnen het bierproductieproces. Ook wordt er dieper ingegaan op de invloed van gisten op het aroma en op de smaak van bieren. Vervolgens wordt een uitgebreid overzicht gegeven van de geschiedenis van pilsbiergisten, alsook hun genetische en fenotypische eigenschappen. Tot slot wordt het toekomstperspectief gegeven omtrent nieuwe pilsbiergisten en worden enkele technieken kort toegelicht die gebruikt kunnen worden om nieuw gegenereerde pilsbiergisten verder te verbeteren.

In hoofdstuk twee werd een *spore-to-spore* kruisingstechniek toegepast om zes specifiek geselecteerde *S. cerevisiae* gisten te kruisen met twee verschillende *S. eubayanus* gisten. Een representatieve set van 31 nieuwe interspecifieke hybridegisten werd vervolgens getest voor

hun temperatuurtolerantie, alsook hun fermentatiecapaciteit en hun aromaproductie in pilsbierfermentaties (op laboschaal en voor sommige gisten op pilootschaal).

Gemiddeld genomen, vertoonden de ontwikkelde interspecifieke hybridegisten een significant betere groei bij lage temperaturen (4°C tot 16°C) in vergelijking met hun respectievelijke *S. cerevisiae*-ouderstammen, alsook een significant betere groei bij hoge temperaturen (30°C tot 37°C) in vergelijking met hun respectievelijke *S. eubayanus*-ouders. Ten eerste zorgde de ruimere temperatuurtolerantie van de nieuwe hybridegisten voor een competitief voordeel in fermentaties bij lage temperaturen ten opzichte van de *S. cerevisiae*-ouderstammen. Daarenboven onderstrepen deze resultaten de capaciteit van interspecifieke hybriden om eigenschappen van de twee verschillende oudersoorten te combineren in één nieuw gegenereerd micro-organisme. Naast de ruimere temperatuurtolerantie, vertoonden de meeste nieuw gegenereerde interspecifieke hybridegisten een verbeterde fermentatiecapaciteit in vergelijking met beide ouderstammen in labo- en pilootschaal pilsbierfermentaties, uitgevoerd bij respectievelijk 16°C en 12°C. Sommige nieuwe interspecifieke gisten produceerden zelfs gelijkaardige ethanolconcentraties als de huidige commercieel toegepaste pilsbiergisten. De aromaproductie van de gegenereerde interspecifieke hybridegisten verschilde tevens significant van de aromaproductie van de huidige, commercieel toegepaste pilsbiergisten, wat het potentieel van deze nieuwe hybridegisten onderstreept voor de productie van nieuwe biertypes, die het vacuüm tussen de goed drinkbare, frisse pilsbieren en aromarijke en diverse bovengistingsbieren kunnen opvullen.

Naast gewenste fenotypische eigenschappen, erfden gegenereerde interspecifieke hybridegisten doorgaans ook enkele ongewenste eigenschappen van de ouderstammen. De meerderheid van de nieuwe gisten waren bijvoorbeeld instaat om ferulazuur om te vormen naar 4-vinyl guaiacol, wat een doorgaans ongewenste kruidnagel-achtige geur en smaak aan het bier toevoegt. Om de productie van deze fenolische *off-flavours* (POF) door de nieuw gegenereerde interspecifieke gisthybriden te onderzoeken, werd er een nieuwe *high-throughput* absorptie-gebaseerde methode ontwikkeld die toelaat om zeer snel het POF-fenotype van honderden gisten in parallel te beoordelen, en dit met een minimum aan werk, ingrediënten of dure apparaten (hoofdstuk drie). De nieuw ontwikkelde test verhoogde niet alleen het aantal gisten dat in parallel getest kan worden, maar reduceerde tevens het

bijhorende kostenplaatje significant. Bovendien vertoonde het een hogere accuraatheid in vergelijking met de bestaande '*state-of-the-art'* methodes.

De nieuw ontwikkelde test werd vervolgens gebruikt in hoofdstuk vier, waar er voor het eerst een succesvolle '*CRISPR-Cas9-based gene editing'* strategie geoptimaliseerd en toegepast werd om POF^-, cisgenetische varianten te creëren van nieuw ontwikkelde en genetisch complexe interspecifieke hybridegisten. Meer precies werd het POF-fenotype van de interspecifieke hybriden veranderd door een natuurlijk veelvoorkomende *non-sense*-mutatie in het ferulazuur decarboxylase-coderend *FDC1*-gen traditionele brouwersgisten te introduceren in het *S. eubayanus* verkregen *FDC1*-allel. Dit gebeurde zonder de introductie van voorheen gerapporteerde *loss of heterozygosity* of andere vormen van *off-target*-activiteit. Daarnaast werden er in uitgevoerde fermentatietesten op labochaal geen fenotypische bijwerkingen gedetecteerd, wat leidde tot de ontwikkeling van aromatisch diverse, maar POF^-, nieuwe pilsbiergisten.

Abstract

With an annual worldwide production exceeding 1.96 billion hectolitre, beer is by far the most produced and consumed fermented beverage. More so, it is considered to be the third most consumed beverage worldwide (after water and tea). Traditionally, beer can be divided into two general styles, namely top-fermented ('ale') beer and bottom-fermented ('lager') beer.

Lager (or Pilsner type) beer accounts for 90% of the total beer production, and is exclusively fermented by *Saccharomyces pastorianus.* Interestingly, this yeast species is not a clean yeast species, but is rather the result of a cross between the brewing and baker's yeast *Saccharomyces cerevisiae* and a cold tolerant wild yeast, *Saccharomyces eubayanus*. The limited genetic diversity of lager yeasts is reflected in the relatively limited influence of the yeast on the aroma profile of lager beer, especially when compared to the immense genetic and aromatic diversity of ale *S. cerevisiae* yeast strains. While the characteristically clean, fresh flavour and aroma of lager beers is one of their most distinctive and praised traits, diversification and differentiation have become increasingly important in today's market.

The development of new lager hybrids may help generating a set of distinct beers that bridge the gap between diverse, aromatic ales and fresh and drinkable lagers, this without the need to change the standard production process or the need for different and more expensive ingredients.

In chapter one, a detailed and comprehensive literature overview is given regarding the role of yeast during industrial beer fermentations and its effect on the aromatic properties and taste of fermented products, as well as the history, genetic- and phenotypic properties of the bottom-fermenting yeast *S. pastorianus* and its future perspectives.

In chapter two, a spore-to-spore breeding strategy was applied, in order to generate novel interspecific hybrids between six carefully selected *S. cerevisiae* and two *S. eubayanus* yeasts. The generated 31 interspecific hybrids were assessed for their temperature tolerance, as well as their fermentation capacity and aroma production in lab- and (for some) pilot-scale lager beer fermentation trials. Overall, generated interspecific hybrids showed a significantly higher growth capacity at low temperatures (4°C-16°C) compared to their respective *S. cerevisiae* parental strains, combined with a significantly higher growth capacity at high temperatures (30°C and 37°C) compared to their *S. eubayanus* parental strains. This broadened temperature

tolerance of the generated interspecific hybrids not only equips them with a competitive advantage compared to their *S. cerevisiae* parent in cold temperature driven fermentations, but it also shows that generated interspecific hybrids can combine interesting phenotypes of both parental species into one organism. Besides a broadened temperature tolerance, most of the generated interspecific hybrids showed hybrid vigour in terms of their fermentation capacity during lager fermentation trials at 16°C and 12°C, with some interspecific hybrids producing similar ethanol levels compared to our best reference *S. pastorianus* strains. Aroma production of the generated interspecific hybrids also differed significantly from the commercially used *S. pastorianus* yeasts, underlining the potential of these novel yeasts for the production of novel beer types that could bridge the gap between easy drinkable lager beers and aroma rich and diverse top-fermented beers.

Apart from only desirable phenotypes, generated interspecific hybrids also inherited some unwanted phenotypes, with the production of phenolic off-flavours (POF) being the most important one. Indeed, the majority of generated interspecific hybrids are POF^+, and are able to convert ferulic acid into its decarboxylated product 4 –vinylguaiacol (4VG), introducing an often unwanted spicy and clove-like aroma in the fermented product. In order to investigate and remediate this unwanted phenotype of novel interspecific hybrids, we first developed a high-throughput absorbance-based screening tool to quickly assess the POF phenotype of hundreds of yeasts in parallel with only a limited amount of labour, consumables or expensive machines needed (chapter three). The developed new assay not only significantly increased the throughput and lowered the cost compared to the current state-of-the-art methods, but also showed an increased accuracy in the determination of the POF phenotype of industrial yeasts.

The novel rapid screening method for POF production in yeast was later on used in chapter four, where a CRISPR-Cas9-based gene editing strategy was developed and applied in order to generate cis-genic POF^- variants of novel generated, and genetic complex interspecific hybrid yeasts, increasing their industrial applicability. Specifically, a natural occurring single nucleotide polymorphism (SNP) in the ferulic decarboxylase coding gene *FDC1*, shared by the vast majority of the current POF^- ale beer yeasts, was selected and introduced into the *S. eubayanus* derived genome of novel interspecific hybrids. Interestingly, the developed CRISPR -Cas9-based gene editing strategy was successful in introducing the selected SNP, without

introducing loss of heterozygosity or other off-target activity, as reported previously when trying to apply CRISPR -Cas9-based gene editing in genetically complex interspecific hybrid genomes. Besides the absence of genetic side effects, no phenotypic side effects were detected, generating aromatically-diverse but POF^- novel lager yeasts.

List of abbreviations

2n	Diploid
3n	Triploid
4EC	4-ethylcatechol
4EG	4-ethylguaiacol
4EP	4-ethylphenol
4n	Tetraploid
4VC	4-vinyl catechol
4VG	4-vinyl guaiacol
4VP	4-vinyl phenol
AD	Anno Domini
A.U.	Arbitrary unit
AAD	Aryl-alcohol dehydrogenases
AATase	Alcohol-O-acyltransferase
ADF	Apparent degree of fermentation
ADH	Alcohol dehydrogenase
BC	Before Christ
BH	Benjamini-Hochberg
cas9	CRISPR associated protein 9
CGH	Comparative genomic hybridization
CoA	Coenzyme-A
CRISPR	Clustered regularly interspaced short palindromic repeats
DMAP	Di-methylallyl mono-phosphate
DMS	Di-methyl sulphide
DMSO	Di-methyl sulfoxide
DSB	Double stranded break
DsDNA	Double stranded DNA
e.g.	exemplī grātiā
EU	European Union
FA	Ferulic acid
FACS	Fluorescence associated cell sorting
FDR	False discovery rate
FMN	Flavin mononucleotide
FTH	Flavour threshold
GM	Genetically modified
GMO	genetically modified organism
HDR	Homology directed repair
HO	Homothallic switching endonuclease
HS-GC-FID	Head space gas chromatography with flame ionization detector
IA	Isoamyl acetate
KW	Kruskal-Wallis
LOH	Loss of heterozygosity
mtDNA	Mitochondrial deoxyribonucleic acid
n	haploid
NA	Not available

NHEJ	Non homologous end joining
NMR	Nuclear magnetic resonance
OD	Optical density
ORF	Open reading frame
PAM	Protospacer adjacent motif
PCA	Principal component analysis
PCR	Polymerase chain reaction
POF	Phenolic off-flavour
PPB	Parts per billion
PPM	Parts per million
QTL	Quantitative trait loci
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	rotations per minute
S.	Saccharomyces
sgRNA	Single guide RNA
SNP	Single nucleotide polymorphism
TTP	Thiamine pyrophosphate
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultra violet
VDK	Vicinal diketon
WGS	Whole genome sequencing

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Introduction

Paragraph 1.4 within this Chapter is adjusted from:

Gallone, B., **Mertens, S.,** Crauwelse, S., Lievense, B., Verstrepen, K.J., and Steensels, J. Genomics and Evolution of Beer Yeasts. In Brewing Microbiology: Current Research, Omics and Microbial Ecology, 1st ed. Caister Academic Press, pp. 145–178 (2017).

And:

Gallone, B., **Mertens, S.**, Gordon, J. L., Maere, S., Verstrepen, K. J., & Steensels, J. (2018). Origins, evolution, domestication and diversity of Saccharomyces beer yeasts. *Current opinion in biotechnology*, *49*, 148-155.

Yeast plays a central role in the production of fermented beverages like beer, wine and sake. Besides the production of its primary metabolites (CO_2 and ethanol), yeast produces a wide variety of secondary aroma-active metabolites like fusel alcohols, volatile esters, carbonyl- and phenolic compounds, fatty acid derivatives and sulphur compounds which highly influence the quality and character of the final product (156).

With an annual worldwide production exceeding 1.96 billion hectolitre, beer is by far the most produced and consumed fermented beverage (154). More so, it is considered to be the third most consumed beverage worldwide (after water and tea). Traditionally, beer can be divided into two major beer styles, namely top fermented ('ale') beer and bottom-fermented ('lager') beer. Top fermented beer is mainly fermented by *Saccharomyces cerevisiae,* which is therefore also known as top fermenting or ale yeast. On the other hand, lager beer, which accounts for 90% of the worldwide beer production, is exclusively fermented by another yeast species, named *S. pastorianus* (52). The current limited genetic diversity of lager yeasts is reflected in the relative limited influence of the yeast on the aroma profile of lager beer (57), especially when compared to the immense genetic and aromatic diversity of ale *S. cerevisiae* yeast strains (155–157). While the characteristically clean, fresh flavour and aroma of lager beers is one of their most distinctive and praised traits, diversification and differentiation have become increasingly important in today's market. Indeed, after a long period of consolidation and homogenization of the global beer market, the craft beer movement is rising. Even in traditional beer countries like for example Belgium, UK or Germany, the total number of microbreweries rose with a staggering 74% within the last five years, which goes hand in hand with an expected increasing market share of craft beer from 2.3% to 11% in the coming years (164). More so, from 2004 until 2009, lager beer consumption in Belgium dropped with 8%, a trend that is still ongoing, as in 2016, it kept decreasing with another 4.3% (186). Interestingly, the changing beer market landscape is mostly caused by changing consumer demands. Indeed, research has shown that consumers besides valuing the price and quality of a product, more and more care about product variety and an increased interest in 'local' products (55). The development of new lager hybrids may help generating a set of distinct beers that bridge the gap between diverse, aromatic ales and fresh and highly-drinkable lagers, which is crucial for brewers to reinsure their position on the current challenging niche-driven beer market (57, 58, 80). In the following paragraphs, a short overview is given of the beer production process,

followed by the different types of yeast used in beer production. Next, the production of the most important aromatic compounds produced during beer fermentation are discussed. Finally, an extensive overview on the history of lager yeast and their genetic and phenotypic characteristics is given.

1.1 Beer production

Beer is traditionally made out of four key ingredients, namely malted barley or other cereals, water, hops and yeast. Each of these components attribute to the final taste and aroma of beer. In short, the beer production process can be summarized in the following scheme (Figure 1, (123)).

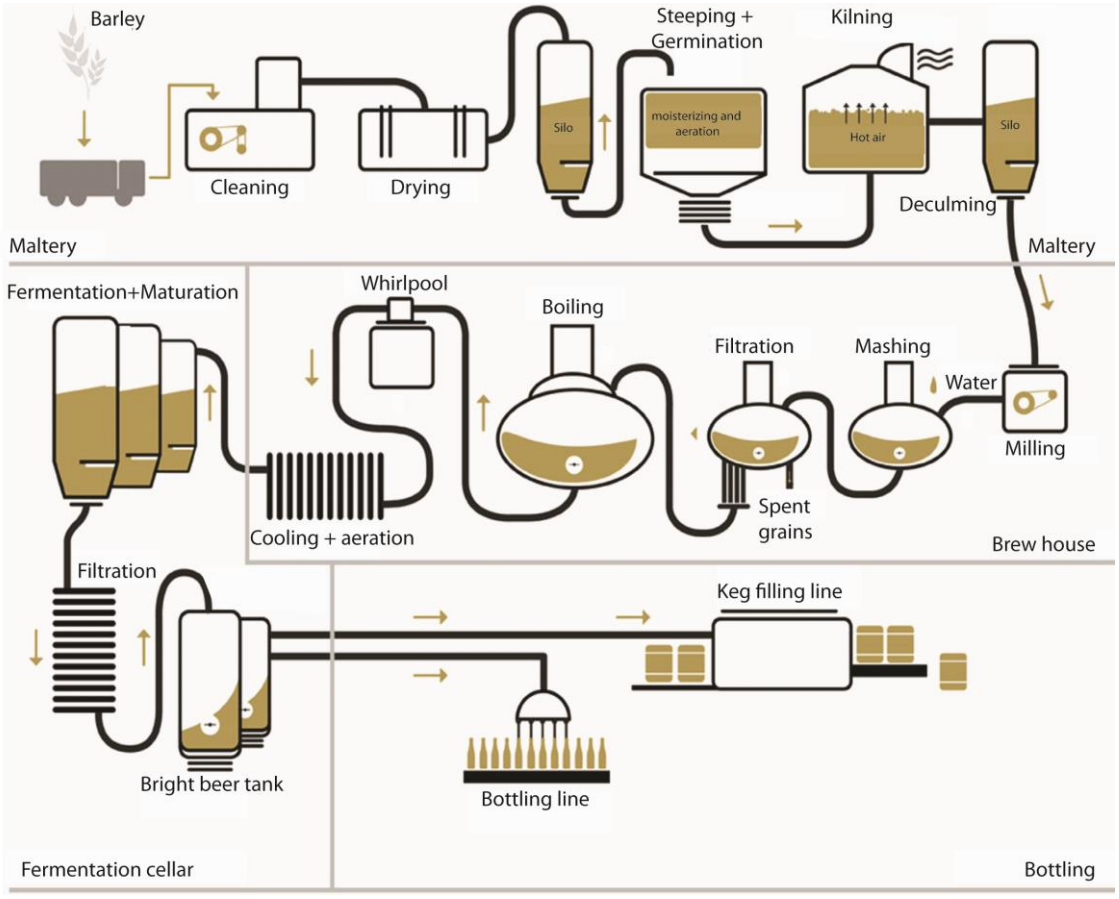

Figure 1: Schematic overview brewing process*. Within the maltery, raw grains are malted before being transferred towards the brewery. Within the brew house, malt and other grains are first milled and mixed with warm water in the mashing-in vessel. Next, wort is filtered and boiled after which it is cooled, aerated and transferred to a fermentation tank. After fermentation and maturation, beer is filtered and bottled or transferred to kegs for consumption (adjusted from* (123)*).*

1.1.1 Malting of barley

The production process of beer starts with the malting of barley or wheat. The main goal is the activation of enzymes within the grain in order to break down starch and other components within the kernels during the mashing (see paragraph 1.1.2) into nutrients and carbon sources suited for yeast fermentation (59, 84).

The malting process starts with the soaking of barley kernels in water combined with periodic aeration (steeping and germination phase), until they sprout. During germination, three important groups of enzymes are formed and activated within the aleuronic layer, each with an important function during the downstream brewing process. Amylases will convert starch, present in the endosperm of the kernels, into fermentable sugars. Proteases break down proteins and form amino acids and short peptides. Lastly, beta-glucanases are formed, which degrade the cell walls of the endosperm, gaining access to the endosperm for other enzymes.

Next, kernels are dried in order to reduce the moister content from roughly 45% to less than 5% (kilning phase). The main goals of the drying step is to arrest further germination of the kernel, as well as arresting the enzymatic activity within the kernels. Drying of the kernels also reduces the risk of possible spoilage and has an effect on the aroma and colour of the final beer (due to Maillard reactions). The last step in the malting process consisted of a deculming step, in which the rootlets or culms are removed from the kernels.

1.1.2 Brewing process

The actual brewing process comprises five steps. The main goal is the conversion of insoluble malt or grain material into a soluble and fermentable extract.

First, malted barley and possibly other grains are milled, after which the formed grist is mixed with warm water (mashing-in). The mash is continuously stirred and kept at precise temperatures and pH (ranging from pH 5.5 to 5.6) for a fixed amount of time, this to ensure proper enzymatic conversion of starch and proteins. Traditionally, a starting temperature of 45°C is used. At this temperature, proteases are activated (active at temperatures ranging from 45°C to 55°C) and degrade proteins to short peptides and amino acids, forming the major nitrogen source for yeast but negatively influencing foam stability of the final beer (84). Next, the mash is heated to 52-54°C, at which starch derived from barley starts to get gelatinized

(barley starch gelatinisation temperatures typically range from 50°C to 60°C), making it accessible for the enzymes.

Subsequently, the temperature is raised to 62-63°C, for 30 minutes up till one hour. At this temperature, β-amylases are active, splicing off maltose from the non-reducing end of starch molecules (Figure 2, (48)). Afterwards, the mash is heated to 72°C, allowing the further breakdown of long chain polysaccharides by α-amylases (± 15-25 minutes). Finally, temperature of the mash is raised to 78°C, stopping nearly all enzymatic activity (mash out) but allowing residual α-amylase activity during the filtering step.

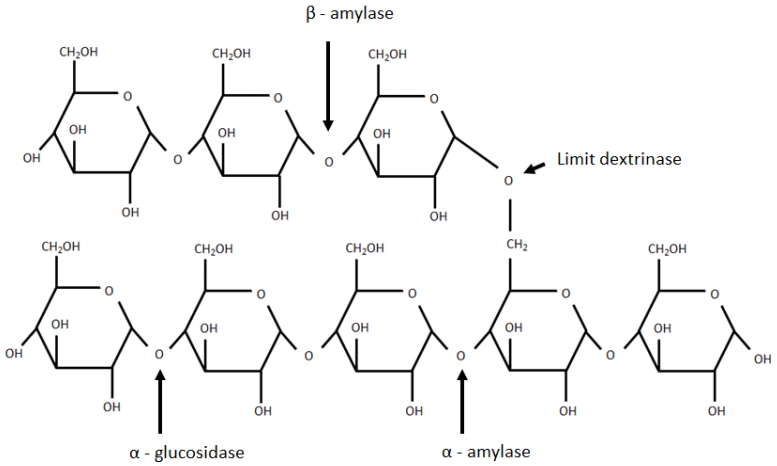

Figure 2: Enzymatic hydrolysis of starch. *α-amylase hydrolyses endo-α-(1-4) bounds of large alpha linked glucose molecules. β-amylases and α-glycosidases hydrolyse α-(1-4) bounds at non reducing ends of starch molecules, resulting in the formation of maltose and glucose respectively. Limit dextrinase is a debranching enzyme, and hydrolyses α-(1-6) bounds. (Adjusted from* (48)*)*

Overall, 75-80% of the total grist gets extracted, of which 60-65% are fermentable sugars (=fermentable extract). The fermentable extract standardly comprises of 12% hexoses (fructose, but mostly glucose), 5% sucrose, 65% maltose and 18% maltotriose (84). The unfermentable extract is a mix of dextrins, proteins and other substances.

Next, the mash is filtered (lautered) to separate the insoluble fraction (spent grains) from the soluble extract. The spent grains are used as animal feed, whereas the remaining extract is transferred to the boiling vessel.

During boiling (± one hour), hops and possibly other spices are added. Added hop components dissolve and isomerization of hop derived α-acids into iso-α-acids takes place, which are the bittering substances in beer. In general, there are three types of hops used for beer brewing,

namely: bitter hops, aroma hops and dual-purpose hops. Bitter hops contain high concentrations of α-acids (6%-16%), and are often added at the beginning of the boil, this to ensure proper α-acid isomerization. Aroma hops have a characteristically high hop oil content (up to more than 1%), which comprises of roughly 200 to 250 different ethereal substances (like for example myrcene, linalool and nonanal) giving the hop its characteristic aroma and flavour (84). Aroma hops are typically only added to the wort at the end of the boil or in the whirlpool, this to reduce the stripping of aroma-active hop components. Aroma hops are also the favourite hops used for dry hopping, where extra hop is added during green beer maturation.

Other effects of wort boiling include protein aggregation (hot trub), water evaporation (causing the wort to get more concentrated) and stripping of off-flavours like dimethyl sulfide (DMS). Last but not least, wort gets sterilized during boiling.

After boiling, wort is transferred to a whirlpool to get rid of the formed hot trub (protein aggregates and insoluble hop components). Finally, the produced wort is cooled, aerated and transferred to a fermenter, where yeast is added.

1.1.3 Fermentation up to final beer

During fermentation, yeast converts fermentable sugars to its primary metabolites CO_2 and ethanol, plus hundreds of different secondary metabolites, influencing the aroma and taste of beer (see paragraph 1.3).

Main fermentation takes about seven to nine days. At the end, most of the yeast is removed, and the green beer is transferred to a maturation tank and stored at low temperatures (5 to -1°C (crash cooling)), for four days (ale beers) up to a couple of weeks (lager beers). At the beginning of maturation, the remaining yeast can still use part of the remaining extract and produce extra CO_2 and ethanol. More so, initial yeast activity leads to the further reduction of off-flavours like diacetyl (paragraph 1.3.4). Therefore, a warm rest at 14°C is often implemented before cooling to -1°C for the maturation of lager beers (79).

During cold storage of the green beer, cold trub is formed, which is an aggregation of proteins and polyphenols. This cold trub is filtered out prior to bottling. In case of bottle refermented beer, extra yeast and sugar is added to the bright beer before bottling, and a secondary fermentation is conducted in a temperature controlled room (18°C to 24°C).

1.2 Brewing yeasts

The core process of fermentation, namely the conversion of carbon sources to carbon dioxide and alcohol, predates the existence of mankind. It can be postulated that the consistent production of fermented beverages was a consequence of a farming or horticultural tradition, and did not evolve until mankind stopped being a hunter-gatherer (during the Neolithic revolution, 8000 B.C.). From then on, beer and other fermented beverages like wine, sake or spirits go hand in hand with human history, where they mainly were produced as a source of nutrition, medicine and as a vital source of uncontaminated water, but also for example as a compensation for labour (57, 69, 77, 89).

Although the production of fermented beverages is deeply rooted in human society, it took until the late 19th century to pinpoint the major causative microorganism that was responsible for this process. Indeed, in the mid-sixteen hundreds Antoni van Leeuwenhoek made a first observation of yeast cells, and 200 years later, Swann and companions proved that yeast was a living organism. 20 more years later, Louis Pasteur confirmed the role of yeast in alcoholic fermentations (11). Shortly after the pioneering work of Pasteur, Emil Christian Hansen was able to obtain the first pure yeast cultures (four in total) during his work in the Carlsberg laboratory in Copenhagen and showed that the usage of one of his pure yeast cultures, named 'Unterhefe Nr.1' (or 'Bottom-fermenting yeast 1') to inoculate sterile wort medium provided the brewers a revolutionary method to reinsure the quality of the produced beer (141). For obvious reasons, it did not take long before clones of this pure yeast culture were introduced in breweries all around the world. The discoveries of Hansen were regarded as revolutionary, and provoked a shift in the beer industry from small-scale, artisanal brewing to large-scale, modern beer production. Besides his pure yeast cultures, also his method for the isolation of these pure cultures had an unprecedented impact on the fermentative industry and beyond, subsequently leading to the discovery, isolation and characterization of more than hundred 'new' yeasts. Since the findings of Hansen, the use of isolated strains as starters for different fermentative industrial processes is available, and is nowadays the rule rather than the exception (38)(31).

Indeed, spontaneous fermentations are only used for the production of a particular type of beer (lambik and geuze beer, a Belgian speciality which production is restricted to the valley surrounding the Zenne valley in Brussels). The production of Lambik beer is a very tedious and

time consuming process, which may take up to three years (119). Typically, these beers are not inoculated with starters during the fermentation step. After boiling, wort is pumped into a large open cupper vessel (coolship), allowing it to cool down overnight and being infected with the wild natural microbiota, which hovers around within the authentic Lambik breweries. Compared to the more conventional fermentation process (paragraph 1.1.3), spontaneous fermentations can be divided into three stages, each with their specific set of core microbes. Within the first month of the fermentation, *Enterobacteriaceae* and oxidative yeasts are mostly present, but get quickly outcompeted by the core microbes of the so called 'alcoholic fermentation', in which mostly *Saccharomyces* yeasts convert sugars to alcohol and CO_2. Besides yeasts, lactic acid (and some acetic acid) bacteria are present, introducing the characteristic sour taste of Lambik and Gueuze beers. After 6 months, the concentration of *Saccharomyces* yeasts drops, and the fermentation gets primarily dominated by *Brettanomyces* yeasts, which contributes to the characteristic barnyard and horse-blanket-like aromas in the beers (9, 20, 119).

Besides spontaneous fermented beer, roughly two other types of beer exist, namely top fermented ('ale') and bottom-fermented ('lager') beer. The ale beer technology, leading to the production of for instance the Belgian Trappist beer, was acquired from the Middle East and Egypt by Germanic and Celt tribes around the first century AD (127). This process is characterized by a top-fermenting process at temperatures ranging from 17-25°C, additionally followed by a short period of maturation and comprises fermentation by the traditional brewer's yeast *S. cerevisiae* or exceptionally by interspecific *S. cerevisiae* X *S. kudriavzevii* yeast hybrids (53, 61).

On the other hand, lager beer is exclusively fermented by another yeast species, named *S. pastorianus* (52). Within the following paragraph, an overview is given of the aroma production of brewing yeasts during beer fermentations. Following is an elaborate paragraph covering the history of lager yeasts and its genetic and phenotypic characteristics (see paragraph 1.4).

1.3 Aroma production during beer fermentation

Besides the production of its primary metabolites (CO_2 and ethanol), yeast produces a wide variety of secondary aroma active metabolites like higher alcohols, carbonyl compounds, phenolic compounds, fatty acid derivatives and sulphur compounds, which highly influence

the quality and character of the final product (Figure 3) (44, 156). Even though, the amount of secondary aroma active compounds produced by yeast is influenced by a wide range of environmental parameters (for an extensive overview, see Dzialo *et all*., 2017 (44)), aroma production mainly depends on the kind of yeast used during the fermentation step (155). Within the following paragraph, a short overview is given of the most brewing-relevant secondary metabolites produced by yeast during fermentation.

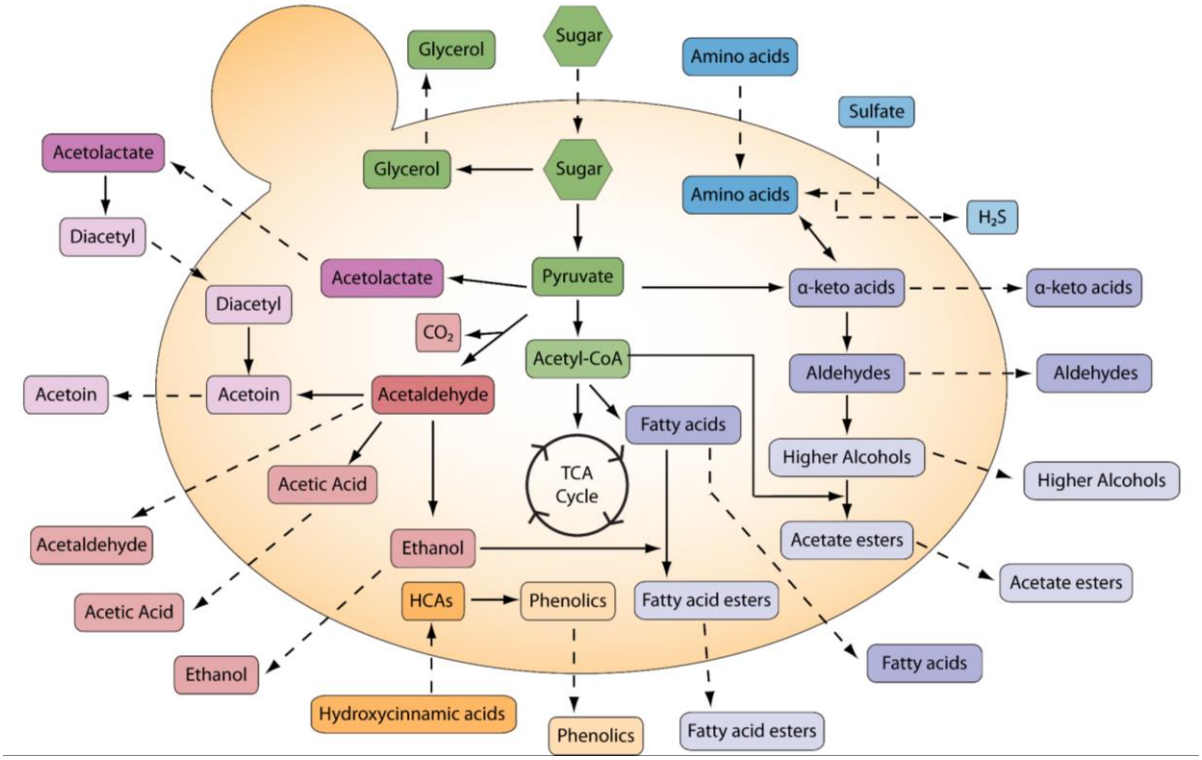

Figure 3: Overview of aroma compound production. *The fermentation of pyruvate (green/red) leads to several carbon-based compounds, including ethanol and carbon dioxide. Pyruvate also feeds into the anabolism of amino acids, leading to production of vicinal diketones (pink). Metabolism of amino acids is responsible for numerous aroma compounds including higher alcohols and esters (purple) as well as sulphur-containing compounds (blue). Additionally, the phenolic compounds are derived from hydroxycinnamic acids found in the media (orange). Compounds shown in darker shades are considered intermediates while lighter shades are aroma compounds. Dotted lines indicate import/export of compounds, solid lines represent biochemical reactions (not indicative of number of reactions). Figure copied from* (44).

1.3.1 Higher alcohols

The major higher- or fusel- alcohols found in alcoholic beverages are isoamyl alcohol (banana, fruity), 2-phenylethanol (flowery, roses), 1-propanol (alcoholic aroma), 1-butanol (alcoholic) and isobutanol (alcoholic) (44). Higher alcohols are formed during yeast fermentation via the Ehrlich pathway, in concentrations ranging from one parts per million (PPM) to a more than

100 PPM. The Ehrlich pathway was first coined by Felix Erhlich in 1907 and comprises three consecutive reactions. First, assimilated amino acids are deaminated into their respective α-keto acid. Four *S. cerevisiae* enzymes (Bat1p, Bat2p, Aro8p and Aro9p) have been shown to be responsible for this transamination reaction, each with their specificity towards particular types of amino acids (27, 76). Next, generated α-keto acids are irreversibly decarboxylated into their respective (fusel) aldehydes (Pdc1, 5 and 6 in *S. cerevisiae*) (44). Created aldehydes can be oxidized into their respective fusel acids, but generally get reduced by various Alcohol dehydrogenases (ADHs) and aryl-alcohol dehydrogenases (AADs) into their respective fusel alcohols.

1.3.2 Esters

Most important esters formed during alcoholic fermentations are 2-phenylethyl acetate (floral, roses), isoamyl acetate (banana, pineapple), isobutyl acetate (fruity), ethyl acetate (solvent, nail polish), ethyl hexanoate (aniseed, apple) and ethyl octanoate (sour apple). Compared to higher alcohols, reported flavour thresholds of esters in beer are lower (on average below 1 PPM, (104)). More so, esters are produced in very low concentrations, ranging from less than 0.1 PPM to a couple PPM. Notwithstanding the fact that esters are generally produced below their respective flavour threshold, synergistic effects between different esters allow them to still have a great effect on the aroma and taste of the final product. Esters are formed in two ways during yeast fermentations. Acetate esters are the result of a condensation reaction of acetyl-CoA and an alcohol, carried out by alcohol-O-acetyl-transferases (AATases) Atf1p and Atf2p (170). So called fatty acid esters are formed via the condensation of acyl-CoA with ethanol by Eeb1p and Eht1p in *S. cerevisiae* (Figure 4) (143). Although the major responsible genes for ester production in *Saccharomyces* yeasts are known, not much is known about their regulation. Therefore, further research is needed in order to unravel this complex, but highly industrial relevant phenotype.

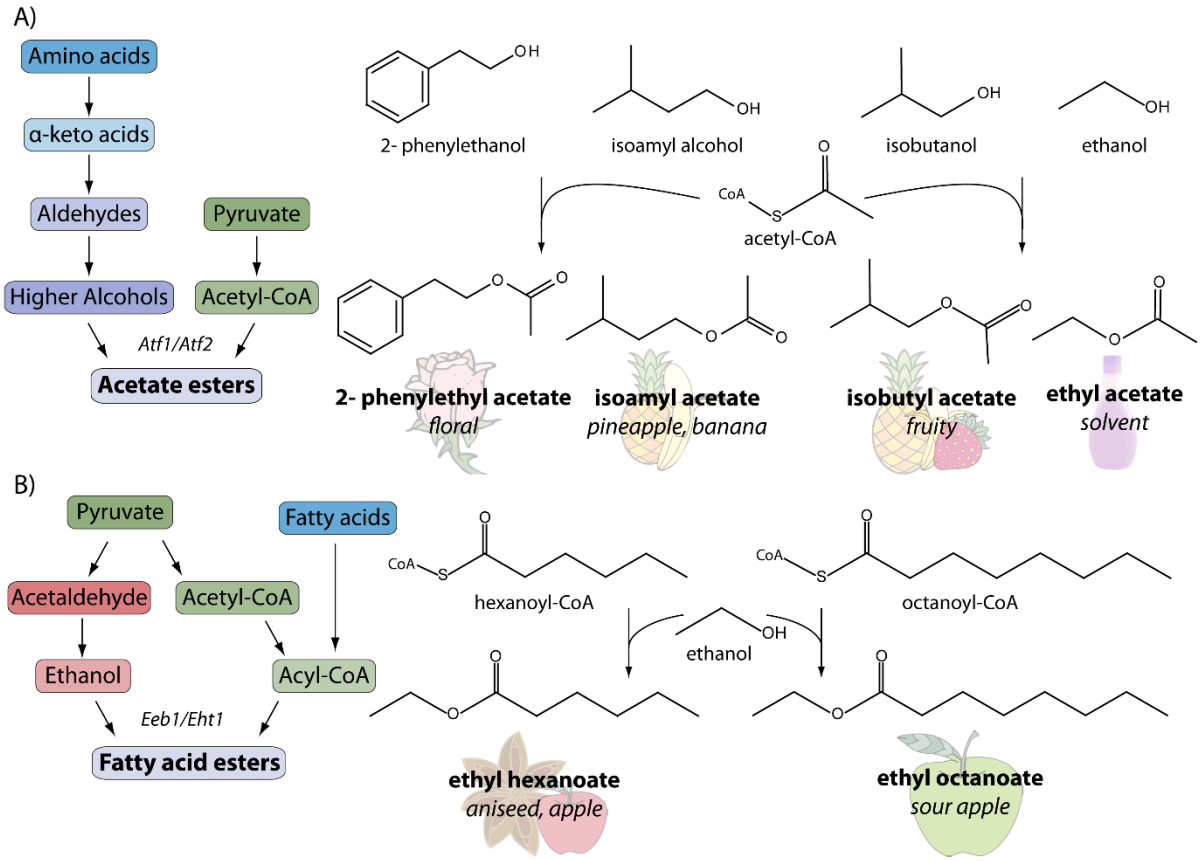

Figure 4: Ester synthesis in yeast. *Left: general scheme of both types of ester production. Esters are the result of condensation reactions between an alcohol and an acetyl/acyl-CoA.* ***(A)*** *Acetate esters are produced through the actions of* Atf1p *and* Atf2p. ***(B)*** *Fatty acid esters are produced by* Eeb1p *and* Eht1p. *Right: examples of some of the most common esters. General aroma descriptors are listed in italics. Figure is copied from* (44).

1.3.3 Sulphur compounds

A wide variety of sulphur containing compounds are formed by yeast including basic thiols (such as H_2S and methanethiol), sulphides (DMS, Dimethyl disulphide (DMDS), …), thioethers and thioesters, sulphur-containing aldehydes and alcohols, as well as large, poly-functional thiols (44). All sulphur containing compounds arise from the catabolism and anabolism of the sulphur-containing amino acids methionine and cysteine. Because levels of these amino acids in nature and in beer wort are relative low, inorganic sulphate is taken up by yeast and through the sulphate reduction sequence (SRS) used for the biosynthesis of both amino acids. Both H_2S and SO_2 are intermediate products of the SRS pathway (75, 153). Whereas the production and release of H_2S is highly unwanted in beer and wine fermentations (‘rotten egg’-odour), sulphite production is often wanted, since is it shown to be an important anti-oxidant, and protects the fermented beverage against bacterial and *Brettanomyces* growth. Also dimethyl

sulfide (DMS – 'cooked vegetables' - odour) production has been linked to cysteine and methionine breakdown. Interestingly, it can also be formed via the reduction of dimethyl sulfoxide (DMSO) by Mxr1p (methionine sulfoxide reductase) during yeast fermentation (44).

1.3.4 Vicinal diketons

Diacetyl (2,3-butanedione) is the most important vicinal diketon (VDK) produced during yeast fermentation. Although its butter-scotch like aroma and taste is very characteristic to for example Czech lager beer, it is highly unwanted in most others (44, 79). Diacetyl, which has a flavour threshold of 50 to 150 parts per billion, is formed during a non-enzymatic decarboxylation of acetolactate, an intermediate formed during valine biosynthesis. Due to the inefficiency of the valine anabolic pathway, excess acetolactate is secreted into the medium, where it is non-enzymatically decarboxylated to diacetyl (44). Later on during the fermentation, it can be reabsorbed by yeast and reduced to acetoin and subsequently to 2,3 butanediol (flavour threshold of 100 PPM, which is 1000 times higher than flavour threshold of diacetyl). VDK's can be formed during the beginning and end of the fermentation. Valine uptake is initially retarded by the presence of amino acids like threonine (part of group A amino acids). Therefore, biosynthesis (and consequently production of acetolactate) happens, causing a first peak in diacetyl formation. If insufficient nitrogen is present in the medium, a potential second diacetyl peak is formed due to renewed valine biosynthesis (79).

1.3.5 Phenolic compounds

Most yeasts (except for the majority of beer yeasts (54)) can convert hydroxycinnamic acids like cinnamic acid, caffeic acid, and most importantly ferulic acid into respectively 4-vinyl phenol (4VP), 4-vinyl catechol (4VC) and 4-vinyl guaiacol (4VG) (Figure 6). This ability of yeast to convert hydroxycinnamic acids into their less toxic decarboxylated form, is believed to help yeast survive in the wild. Phenolic off-flavours (POF), and most notably 4VG, are regarded as the most unwanted off-flavours possibly produced during beer or wine fermentations (except for Belgian wit beers, some Belgian speciality ales and the German Hefeweizen beers, where its clove-like aroma is part of the beer style (167)) (165). POF's are characterized by a low flavour threshold (0.2 to 0.4 PPM) and are characterized by a medicinal or clove-like aroma. 4VG in beer is mainly formed by yeast via the decarboxylation of ferulic acid (167). Ferulic acid can be found in plant cell wall material, associated with polysaccharides. During the brewing

process, ferulic acid is both extracted and solubilized by hydrolases from the malt into the wort (33). In *Saccharomyces* yeasts, two genes (namely *PAD1* (728bp) and *FDC1* (1511bp), both situated within the right subtelomeric region of chromosome IV for *S. cerevisiae*) are required for 4VG production. *PAD1* encodes for a flavin prenyltransferase (26.7 kDa), which catalyses the formation of a prenylated flavin mononucleotide (prFMN), acting as a cofactor required for the ferulic acid decarboxylase, encoded by *FDC1* (56.1 kDa)(29, 114). Decarboxylation of organic acids like ferulic acid are inherently difficult. First of all, most organic acids are very stable molecules, and heterolysis of the C-C bound, resulting in the release of CO_2, requires the generation of a highly nucleophilic carbanion intermediate (Figure 5A). At ambient conditions, the decarboxylation of organic acids only occurs when the carbanion intermediate is stabilized. In nature, different decarboxylases have evolved, reflecting a wide variety of chemical approaches to stabilize or bypass the formation of this carbanion intermediate. Most of them depend on the use of cofactors, ranging from metal ions to organic molecules such as for example thiamine pyrophosphate (TPP; involved in pyruvate decarboxylation) (5, 101, 129). Only recently, it is shown that the decarboxylation of hydroxycinnamic acids requires a new type of cofactor, namely a prenylated flavin mononucleotide. *PAD1* encodes for a flavin prenyltransferase, producing a prenylated FMN by transfering a prenyl group from di-methylallyl mono phosphate (DMAP) (Figure 5B). The formed reduced prFMN is further matured within the active pocket of *FDC1* to its active iminium form ($prFMN^{iminium}$).

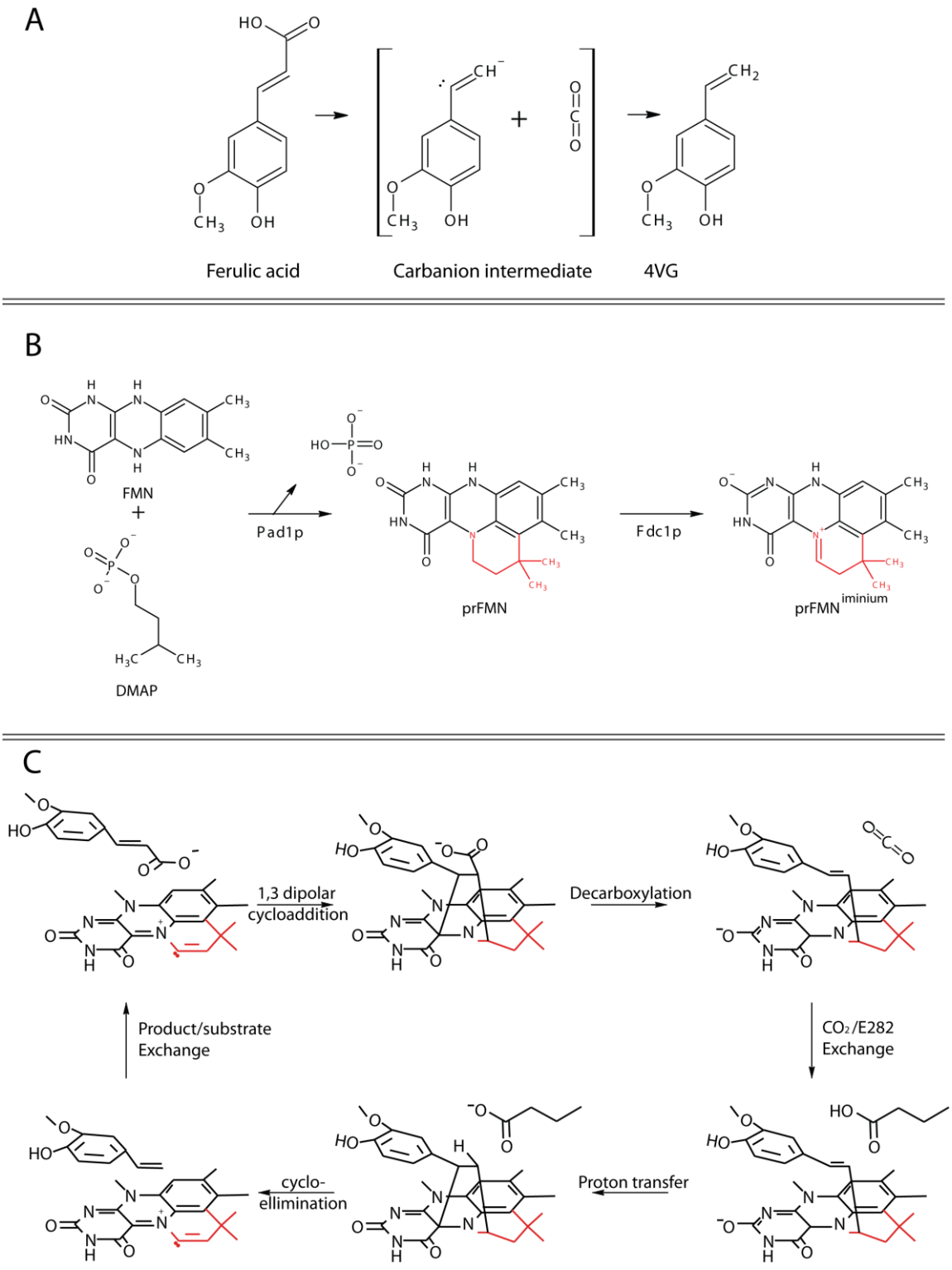

Figure 5: Pad1p and Fdc1p activity. *A) Ferulic acid decarboxylation requires the formation of a carbanion intermediate. B) Prenylation of FMN by Pad1p. Pad1p transfers a prenyl group from dimethylallyl mono phosphate (DMAP) to FMN. The formed reduced prFMN is further modified within the Fdc1p active pocket, where the active form of prFMN, namely prFMN^{iminium}, is formed. C) Decarboxylation of Ferulic acid to 4VG. prFMN^{iminium} and ferulic acid undergo a 1,3 dipolar cycloaddition in the active pocket of Fdc1p, which allows the decarboxylation to take place. Next, CO_2 is replaced by the glutamine amino acid on position 282 of* Fdc1p*, which is followed by a proton transfer and a cyclo-elimination, allowing the release of 4VG from the active pocket.*

Within the active pocket of Fdc1p, the hydroxycinnamic acid is positioned above the formed prFMN^{iminium} in a confirmation known as pi-stacking, where aromatic rings are positioned nicely on top of each other. Only recently, a proposed mechanism on how the exact decarboxylation happens was released (Figure 5C, for more information see (101)). First, a process called azomethine ylide 1,3 dipolar cycloaddition between the prFMN^{iminium} cofactor and the hydroxycinnamic acid takes place, destabilizing the organic acid structure, allowing the following decarboxylation. Next, the formed CO_2 molecule is exchanged for the glutamic acid on position 282 (E282). Finally, a proton transfer followed by a cyclo-elimination resets the cofactor and allows product/substrate exchange.

Some wild yeasts (like for example *Brettanomyces*) or bacterial species can subsequently reduce 4VP, 4VG and 4VC into respectively 4-ethylphenol (4EP), 4-ethylguaiacol (4EG) and 4-ethylcatechol (4VC) by a vinylphenol reductase (167). 4VG and 4EG are associated with more pleasant clove-like or spicy aromas, whereas 4VP and 4EP aromas are more medicinal and 'Band-Aid'-like (44). Because *Saccharomyces* yeasts lack this reductase activity, the presence of 4EG and 4EP in an fermented beverage could point towards a possible bacterial or *Brettanomyces* contamination (157).

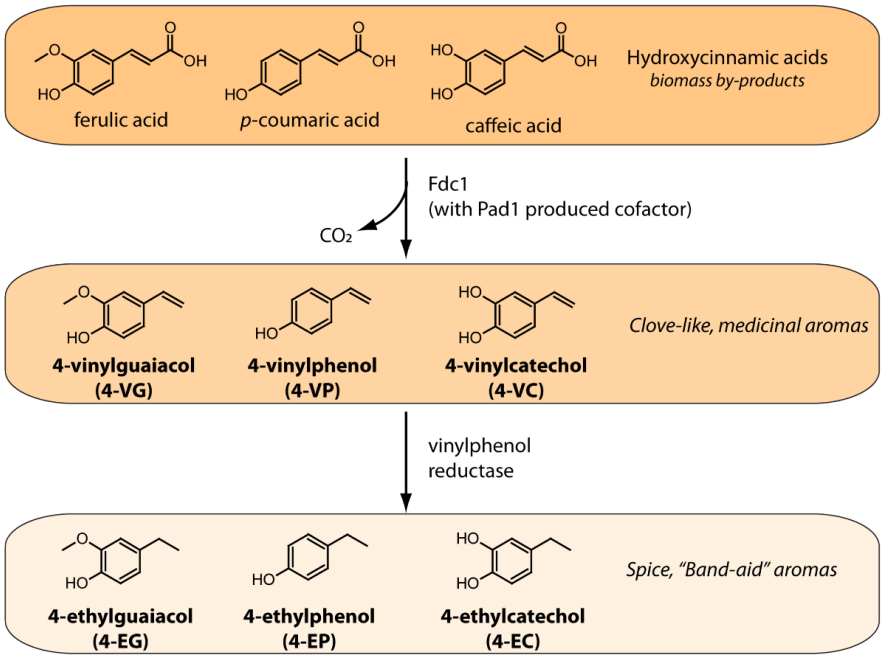

Figure 6: Production of phenolic compounds. *Hydroxycinnamic acids are released during the brewing process. Yeast cells can decarboxylate hydroxycinnamic acids to less harmful forms through the actions of* Fdc1p. Fdc1p *requires a prenylated FMN cofactor which is formed by* Pad1p. *The compounds are then secreted and can be further reduced by a vinylphenol reductase, typically by contaminating yeast or bacterial species. Figure copied from* (44).

1.4 *Saccharomyces pastorianus* is an interspecific hybrid yeast

As mentioned earlier (paragraph 1.2), lager beer currently account for more than 90% of the global beer market (154).They are typically fermented at a lower temperature (8 to 15°C), after which a period of cold storage (i.e., lagering, a traditional practice vital for sensorial quality) is performed. It is believed that the lager beer production process originally was introduced in the 16th century in Bavaria (Germany), when brewing became legally restricted to wintertime (at colder temperatures) to minimize the microbial spoilage of Bavarian beer (also known as the Bavarian law). Later, the advent of refrigeration in the 19th century enabled lager brewing throughout the whole year (57, 77, 136). Because of the high appreciation of this type of beer, it quickly spread around the globe. The yeasts used in this practice typically sink to the bottom (and not rise to the top) of the fermentation vessel towards the end of the fermentation, and are therefore often called "bottom-fermenting yeasts". Interestingly, this bottom-fermenting phenotype was described very quickly after the dawn of lager beer brewing in Nuremberg, a town in the state of Bavaria (21, 57, 69, 77, 151). In the 19th century, ground-breaking work by Louis Pasteur established that this phenomenon was caused by yeast (11, 125, 126, 140), and the species name *S. pastorianus* was first coined by the German scientist Max Rees in 1870 as a tribute to Pasteur's work in the field. Emil Christian Hansen isolated shortly thereafter the first pure yeast cultures from lager beer fermentations during his work in the Carlsberg laboratory in Copenhagen. He classified the three isolated pure yeast lineages as separate species; one as *S. pastorianus*, one as *Saccharomyces carlsbergenesis* ("Unterhefe Nr. 1"), and one as *Saccharomyces monacensis* ("Unterhefe Nr. 2") (11, 107, 141). After Hansen's findings, starter cultures became general practice in the breweries and bottom-fermenting yeast was classified as *S. carlsbergensis*, disregarding the earlier classification of bottom-fermenting yeast as *S. pastorianus* by Max Rees. However, genetic analysis of the various isolates in 1985 showed that the type strains of *S. carlsbergensis, S. monaccensis,* and *S. pastorianus* (the original isolates from Hansen) were almost identical, resulting in the reclassification of all bottom-fermenting yeasts to *S. pastorianus* (21, 57, 77, 134, 151, 182). Furthermore, recent research has revealed that *S. pastorianus* is not a pure species at all, but instead an interspecific hybrid of *S. cerevisiae x Saccharomyces eubayanus.* Nevertheless, in practice the species name *S. pastorianus* is still used to denote this lineage of interspecific hybrids.

Because of its industrial importance, much research has been dedicated to the characterization of the lager yeast genome. This led to novel insights in the peculiar genome of this species, provided clues about its origin and shed light on some evolutionary processes that enabled this species to thrive in lager beer fermentation.

1.4.1 Hybrid nature of lager yeast

The physiology of lager yeasts differs fundamentally from the physiology of other brewing yeasts (11, 126, 140). One of the most peculiar differences is the inability of *S. pastorianus* to sporulate and form viable spores, a property that is still present in many ale-type (and other *S. cerevisiae*) yeast strains (3, 116, 155). This inability to form viable offspring is a trait typically encountered in interspecific cross-breeding, e.g., mules (horse x donkey) or ligers (lion x tiger). Indeed, early genetic analysis showed that *S. pastorianus* harboured genetic material of (at least) two different species, and was thus not a clean yeast lineage, but rather the result of a hybridization event between *S. cerevisiae* and another (non-*cerevisiae*) *Saccharomyces* species. The first molecular evidence of the hybrid nature of lager yeast was obtained by a technique, called kar-mediated single chromosome transfer (118), and revealed that the chromosomes of lager yeasts could be divided into three types: i) homologous (*cerevisiae*-like) chromosomes, ii) homeologous (non-*cerevisiae* like) chromosomes, and iii) mosaic chromosomes, i.e. chromosomes composed of both homologous and homeologous segments. Later, these findings were confirmed by several DNA hybridization experiments (e.g. by using southern blotting or *S. cerevisiae*-specific gene arrays) (42, 128, 162, 184).

By comparing the DNA sequence of 11 independent loci, Dunn and Sherlock further suggested that the *S. cerevisiae* parent of *S. pastorianus* was closely related to ale-type *S. cerevisiae* strains, and not to wild isolates or strains used in other fermentation industries (42). This result was in line with the previous work in which allelic variation in 12 microsatellite loci of 651 diverse *S. cerevisiae* and 15 *S. pastorianus* strains was investigated (87).

The first research suggesting a potential origin of the non-*cerevisiae* part of the lager genome was published in 1985 (169). Using DNA-DNA hybridization methods, the authors revealed a similarity of 72% between the non-*cerevisiae* moiety of the lager yeast CBS1513 (at that time classified as *S. carlsbergensis*) and *S. bayanus*, a cold tolerant species commonly encountered in wine fermentation environment. This finding was later confirmed by PCR/RFLP analysis of

48 genes of the same lager yeast strain (137). Interestingly, this hypothesis was later questioned when it was established that part of the *S. pastorianus MET2* gene showed a significant sequence difference with the corresponding *S. bayanus* sequence, suggesting a closely related *Saccharomyces* species, rather than *S. bayanus*, as the non-*cerevisiae* parent (64). In 2009, the analysis of the first whole-genome sequence of a lager strain (Weihenstephan 34/70) confirmed this hypothesis. By aligning annotated Open Reading Frames (ORFs) of the lager brewing strain Weihenstephan 34/70 to annotated ORFs of *S. cerevisiae* S288c and *S. bayanus* CBS7001 reference genomes, the *cerevisiae*-type subgenome showed very high similarity to *S. cerevisiae* S288c (> 99 %) as opposed to the *bayanus*-type subgenome, exhibiting lower sequence identity with *S. bayanus* CBS7001 (average of 92.7%) (116). Moreover, the authors identified eight genes in the lager yeast genome that were not present in the genomes of the *S. cerevisiae* yeast S288c nor in the genome of the *S. bayanus* yeast CBS7001, further indicating that probably a different, yet closely related species is the second lager yeast parent.

In 2011, Argentinean researchers sampling for cold-tolerant *Saccharomyces* yeasts in the Patagonian forest stumbled upon a new *Saccharomyces* species (89). The draft genome obtained through whole genome sequencing (WGS) of this species (dubbed *S. eubayanus*), showed a remarkable high degree of similarity (99.56%) to the non-*cerevisiae* portion of the lager yeast genome, indicating that this species is very likely the missing link in the *S. pastorianus* origin. The authors further suggest a possible scenario where the initial hybridization event between a diploid *S. cerevisiae* cell and a diploid *S. eubayanus* cell gave rise to an allotetraploid hybrid (the original *S. pastorianus* strain), which was subsequently subjected to extensive genome rearrangement and mitotic recombination, resulting in loss of heterozygosity and recombinant chimeric chromosomes. Since these adaptations occurred in the highly selective and man-made environment of (lager) beer fermentation, they considered this the “domestication” of lager yeasts (Figure 7).

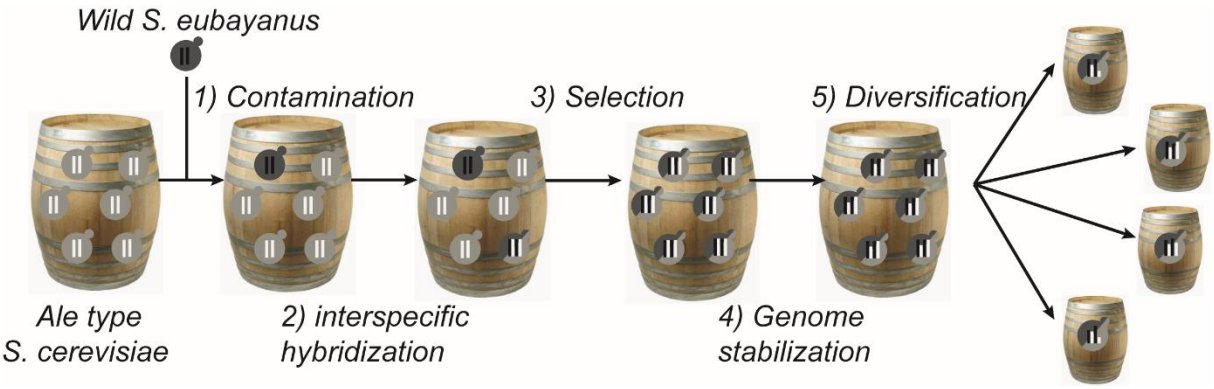

Figure 7: The origin of lager yeast. *Current hypothesis about the origin of lager yeast involves (1) a contamination of ancient Bavarian fermentations (suggested to be originally conducted by ale yeast S. cerevisiae) by a wild S. eubayanus yeast contaminant; (2) a rare interspecific hybridization event between both yeast species; (3) selection of the interspecific hybrid due to its likely ability to combine the fermentation capacity and ethanol tolerance of its S. cerevisiae parent with the cold tolerance of its S. eubayanus parent; (4-5) followed by genome stabilization and diversification (domestication) of the ancient S. pastorianus yeast to its current conformation. This whole process happened at least once or twice in history (see later), giving rise to the two groups of lager yeasts (Saaz and Frohberg types).*

However, several questions still remain unanswered. While *S. eubayanus* was originally discovered in Argentina, it is rather unlikely that lager yeasts originated in South America. Initially, Libkind and coworkers hypothesized that the South American *S. eubayanus* strain was introduced in Europe via the transatlantic travel between Europe and America (89, 132). However, while transatlantic travel was only established after Columbus' first voyage to the new world and the first reports of the bottom-fermenting phenotype predate Columbus' travels, lager brewing yeasts most likely originated earlier (probably in the early 1400s in Bavaria) (21, 57, 69, 116, 151). More recent discoveries of genetically distinct lineages of *S. eubayanus* in other parts of the world (North America, China and New Zealand) suggest that *S. eubayanus* is not unique to South America (19, 132). Moreover, genetic evidence suggests that the non-*cerevisiae* moiety of the Weihenstephan 34/70 is more closely related to a *S. eubayanus* lineage isolated in Tibet (sequencing of 12 loci indicated a 99.82% similarity to the non-*cerevisiae* moiety of lager yeast of the Asian *S. eubayanus* isolate, compared to the 99.56% similarity obtained by WGS with the Argentinean isolate described by Libkind (89)), suggesting that this lineage is more likely the direct ancestor of this lager yeast (19). Therefore, it is now hypothesized that *S. eubayanus* made his way to Europe via the 2000 year old Silk Road. Nevertheless, *S. eubayanus* is up till now not yet discovered in Europe and it could be that this yeast species occupies a highly specific niche in Europe and still awaits discovery (57).

However, the question of when and how *S. eubayanus* got into contact with the lager brewing environment remains open. It is hypothesized that this event took place five to six hundred years ago, triggered by a law enforcing brewing at cold temperatures in Bavaria. The non-*cerevisiae* parent (*S. eubayanus*) is thought to occur as a wild yeast contaminant around the brewing environment, and being better equipped to withstand the fermentation conditions in cold temperatures compared to the native ale yeasts. However, phenotypic analysis of the first two *S. eubayanus* strains isolated showed an inferior fermentation profile of *S. eubayanus* compared to *S. cerevisiae* (e.g., it is unable to ferment maltotriose, shows a lower ethanol tolerance, and produces an inferior aroma profile), prohibiting its use as starter cultures for lager beer fermentation. An interspecific hybridization event (between the *S. eubayanus* contaminant and the ale-type *S. cerevisiae*), that probably happened within the brewing tank, resolved the shortcomings of both species, and resulted in a hybrid species (*S. pastorianus*) that possessed the combined advantage of cold tolerance and a good fermentation capacity. This species was therefore able to outcompete its parental strains in lager beer fermentations, and was in this way (unintentionally) selected by the brewers, as traditionally part of the fermented beer was used to inoculate the next batch (57).

1.4.2 Lager yeasts can be divided into two genetically and phenotypically distinct lineages

Over the years, the interspecific hybridization between *S. cerevisiae* and *S. eubayanus* yeasts probably occurred numerous times. However, to date, only two lineages of lager yeast remain present in industry. These two archetypes are referred to as “Saaz” or “group I” and “Frohberg” or “group II” type *S. pastorianus* yeasts (42, 49, 57, 58, 94, 178). These names refer to the work of Paul Lindner (1909), who reported the isolation of two individual *S. pastorianus* yeast lineages, which he named “Saaz” and “Frohberg” after the locations in Bohemia and Germany in which these strains were originally used (57, 58, 90).

Today, a clear trend exists between the lager yeast archetype and the country in which these yeasts are used. Group I (Saaz type) are mainly used in Czech breweries, as well as the Carlsberg brewery in Denmark, whereas Group II (Frohberg type) are more widespread in other European and North-American breweries (42). While these archetypes show many similarities, there are some interesting genetic and phenotypic differences as well.

1.4.3 Genetic differences

The separation of *S. pastorianus* yeasts into two genetically distinct groups was firstly suggested based on RFLP analysis and the analysis of transposon sequence distribution among different *S. pastorianus* yeasts (94, 96). Later, this hypothesis was confirmed by the use of array Comparative Genomic Hybridization (array CGH) and DNA sequencing of the adjacent and intronic regions of 11 intron-containing genes (42). In the same experiments, it also became clear that both archetypes did not share a common ancestry. Initially, it was predicted that Saaz-type yeasts originated from a haploid-haploid hybridization event, whereas Frohberg-type yeasts derived from a diploid-haploid hybridization event, where the diploid moiety originates from the *S. cerevisiae* parent (21, 42). However, recent WGS analysis revealed that both Weihenstephan 34/70 (a Frohberg-type yeast) and CBS1513 (Unterhefe I, a Saaz-type yeast) are allotetraploid strains, both originating from a diploid-diploid rare mating event between *S. cerevisiae* and *S. eubayanus* (116, 178). Interestingly, the two strains differed remarkably in the ratio in which both parental strains were retained in the genome: while Weihenstephan 34/70 harboured a tetraploid genome of 23.6Mb, with an approx. 1:1 ratio of both parental strains (36 different chromosome structures, 64 chromosomes in total), the genome of CBS1513 was much smaller (19.5Mb), and the *S. cerevisiae* parental strain was underrepresented compared to *S. eubayanus* (allotriploid (3n-1) with 29 different chromosome structures, 47 chromosomes in total). This interesting parental imbalance in the CBS1513 genome was shown to be caused by a large loss of the *S. cerevisiae* genome, including three complete *S. cerevisiae* chromosomes (VI, XI and XII). This is in line with previous findings based on PCR-RFLP (137) and array CGH (42). Additionally, the CBS1513 genome was characterized by numerous large regions of loss of heterozygosity in chromosomes originating from *S. cerevisiae*, resulting in homozygous sequences derived from the *S. eubayanus* parent. Indeed, it was shown that approximately 1.44 Mb of *S. cerevisiae*-derived DNA got replaced by its *S. eubayanus* complement on four different chromosomes (chromosome IV, XIII, XV and XVI), whereas the opposite was only true for 0.22 Mb.

More recently, Okuno et al. (2016) analysed in more detail the genetic composition of ten lager yeasts (five Saaz type: CBS1503, CBS1513, CBS1538, CBS1174, CBS2440 and five Frohberg type W34/70, CBS1483, CBS1484, CBS2156, CBS5832) using Illumina next generation sequencing (120). The ploidy of the strains was estimated by mapping the obtained paired-

end reads onto the genomes of both parental species. *Saccharomyces cerevisiae* derived chromosomes in Saaz type yeasts were haploid or missing, whereas most *S. eubayanus* derived chromosomes appeared to be diploid or triploid. Moreover, partial or whole deletions of *S. cerevisiae* derived chromosomes were observed frequently (e.g. deletions of the right arm of chromosome IV, left arm of chromosome XIII and the entire chromosome XII were common in all five sequenced Saaz type lager yeasts). In contrast, the genome of Frohberg type lager yeasts was composed of a haploid or diploid set of *S. cerevisiae* derived chromosomes whereas the ploidy of *S. eubayanus* derived chromosomes ranged from haploid to triploid. The observed chromosomal imbalance implied that the five Saaz type *S. pastorianus* strains, together with Frohberg type lager yeast CBS2156 can be regarded as being triploids, with a total genome size ranging from 14.4 Mb to 19.2 Mb and that Frohberg type lager yeasts (except for CBS2156) are tetraploids (120). Analysis of the genome structure of *S. pastorianus* pinpoints to a complex evolutionary history and allows inference on the origin of the new species.

Throughout the years, there has been some uncertainty about whether both *S. pastorianus* archetypes originate from a single hybridization between *S. cerevisiae* and *S. eubayanus*, or if the Saaz-type and Frohberg-type lager yeasts are derived from two independent hybridization events (For a review see (109)). Three main hypotheses for the *S. pastorianus* origin have been proposed, and different analyses have provided support for each. The most widespread hypothesis involves two completely independent hybridization events, each involving a different domesticated ale-type *S. cerevisiae* and a different wild *S. eubayanus* strain (Figure 8a). This hypothesis is supported by phylogenetic analysis, where the relative branch lengths for the S. *cerevisiae* and the *S. eubayanus* subgenomes are significantly different between the groups (8), as well as by the pattern of loss or retention of subtelomeric regions in the *cerevisiae* part of the lager yeast genome, which are very different in Saaz and Frohberg yeasts (110). For example, Monerawela and coworkers (108) showed that the Frohberg strains originated from a stout-type yeast, while the *S. cerevisiae* moiety of the Saaz strains had the highest similarity with Foster O-like ale strains (an Australian ale yeast with European roots).

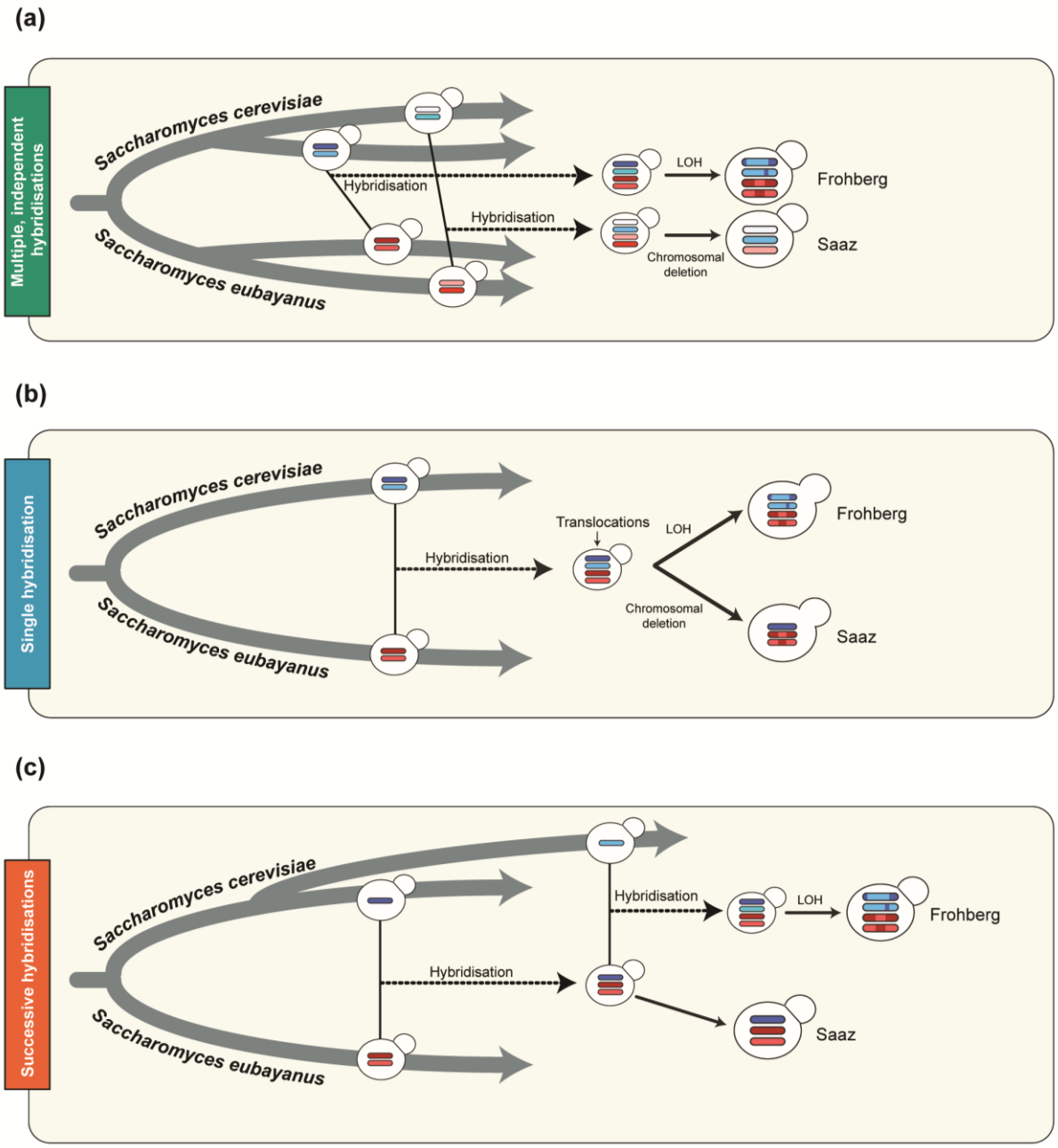

Figure 8: Current models for the origin of Frohberg and Saaz lineages. *a) Frohberg and Saaz groups originated from at least two independent hybridisation events between distinct diploid S. cerevisiae and diploid S.eubayanus parental strains. b) Frohberg and Saaz groups originated from a single hybridisation event between a diploid S. cerevisiae and a diploid S. eubayanus. Translocations occurred in the ancestral hybrid prior to the divergence of the Saaz and Frohberg lineages and are shared between the two groups. After hybridisation, the Frohberg lineage experienced loss of variation between intra-homologous chromosomes in the S. cerevisiae subgenome via loss of heterozygosity (LOH)* (120) *and the Saaz lineage lost roughly half of the S. cerevisiae derived chromosomes. c) Frohberg and Saaz groups originated from at least one shared hybridisation event between a haploid S. cerevisiae and a diploid S.eubayanus. The triploid ancestral hybrid further diverged into the Saaz lineage, and the Frohberg lineage arose by another hybridization event with a distinct haploid S. cerevisiae* (120).

However, other studies have identified several *S. eubayanus/S. cerevisiae* translocations that share identical breakpoints within the subgenomes of both Saaz and Frohberg lineages (68, 178) (Figure 8b). Although it has been argued that identical breakpoints could have resulted from i) independent events at recombination hotspots or fragile sites in *Saccharomyces* chromosomes, ii) events that occurred in one of the parental strains prior to the hybridization (8), they might also indicate a shared hybridization event prior the divergence in the distinct lager lineages.

A further hypothesis suggests a combination of the two scenarios; a single hybridization event between a haploid *S. cerevisiae* and a diploid *S. eubayanus* that led to an ancestral Saaz-like hybrid, followed by a second hybridization with a distinct haploid *S. cerevisiae* isolate that led to a Frohberg-like ancestral hybrid (120) (Figure 8c).

In addition, the mtDNA of *S. pastorianus* showed some interesting trends. In a study on 22 different lager yeasts by RFLP analysis (using four different restriction enzymes), all lager yeasts tested showed a similar uni-parental inheritance of the mtDNA of their non-*cerevisiae* parent (138). These findings were later confirmed in the work of Dunn and Sherlock (where none of the 17 tested lager yeasts seemed to contain *S. cerevisiae*-derived mtDNA (42) and the WGS of Weihenstephan 34/70 (116). This suggests that *S. eubayanus* mtDNA might harbour one or more genes that provide a competitive advantage for *S. pastorianus* in a lager beer environment. This theory finds further support in the work of Gonzalez and coworkers, who also observed a similar uni-parental inheritance of mtDNA in natural interspecific hybrids between *S. cerevisiae* and the cold-tolerant species *S. bayanus* and *S. kudriavzevii*, discovered in European wine fermentation environments (60). However, the genes causing this trend are yet to be identified.

1.4.4 Phenotypic differences

The two lager yeast archetypes were originally described in 1909 (90), but the first systematic phenotypic screening of these groups was only published recently (57, 96, 105, 178). In these studies, several remarkable differences were revealed.

First, Saaz-type yeasts showed a higher growth capacity at 10°C than Frohberg-type yeasts, suggesting that Saaz-type yeasts harbour a higher tolerance towards cold temperatures (58, 178). Second, Frohberg-type yeasts showed a faster fermentation profile and higher degree

of attenuation in 22°P fermentation at 15°C (58), 14°P fermentation at 14°C (178), and 12°P fermentation at 16°C (105). This remarkable difference was explained by the incapability of Saaz-type lager yeasts to efficiently metabolize maltotriose (58). Third, Saaz-type yeasts showed lower cell viability and formed more respiration-deficient 'petite' cells at the end of fermentation. These phenotypes are generally undesired in lager yeasts, since lager yeasts are traditionally reused for seven to 21 consecutive fermentation batches, and thus require high phenotypic stability. Finally, the aroma profile of both lager yeast archetypes also differed significantly. In general, Frohberg-type yeasts produced higher concentrations of ethyl acetate, isoamyl acetate, and isoamyl alcohol, but less acetaldehyde (Chapter 2). These phenotypic differences may partly explain why Frohberg-type lager yeasts are generally preferred over Saaz-type lager yeasts in today's beer industry.

Although lager yeasts can be divided into two groups, the genetic and phenotypic diversity within these groups is very limited (58, 178). Because of a decreasing consumption of lager beer and the change of the beer market into a niche market, the development of novel lager yeasts might give an new tool for brewers to create novel beer, and by this securing their position on the beer market.

1.4.5 Future prospects of lager yeasts

The earlier described limited genetic and phenotypic diversity of lager yeasts (especially when compared to the immense genetic and aromatic diversity of ale *S. cerevisiae* yeast strains) has inspired several researchers to develop new and more diverse lager yeasts (for the most recent review, see (57)), and formed the start of this PhD project (Chapter 2).

More precise, the recent discovery and isolation of *S. eubayanus* lent the opportunity to develop new interspecific hybrids between *S. cerevisiae* and *S. eubayanus* in the lab, providing a powerful tool to generate strains with superior brewing properties (66, 80, 105). Indeed, the development of interspecific hybrids has proven to be an efficient approach to develop novel yeast variants with enhanced characteristics for wine- and beer-making. Typically, an industrial strain of *S. cerevisiae* yeast is crossed with a wild, non-*cerevisiae* member of the *Saccharomyces* sensu stricto complex, such as *S. bayanus* (100, 146–148), *S. kudriavzevii* (15, 131), *S. uvarum* (41, 133), *S. mikatae* (16), or *S. eubayanus* (66, 80, 105). The first attempt to generate new lager yeasts was established through pair-wise mass mating of ale type *S.*

cerevisiae yeasts and a *S. bayanus* yeast strain. Resulting hybrids showed a higher fermentation capacity than both their respective parental strains, and some showed a similar fermentation capacity to their control bottom-fermenting yeast, already highlighting the potential of newly formed interspecific yeast hybrids for lager beer brewing (146). More recently, two papers were published in which a similar, auxotrophic marker-assisted mass-mating strategy was used to generate four interspecific yeast hybrids between *S. eubayanus* type strain CBS12357 (wild isolate from Patagonia (89)) and *S. cerevisiae* strains IMK439 (66) or VTT-A81062 (80). The hybrids inherited interesting properties of both parental strains (cold tolerance, maltotriose utilization, and strong flocculation) and showed hybrid vigour for several traits, such as fermenting speed and fermentation capacity in lab-scale lager beer fermentation tests (66, 80).

Within this PhD, a more elaborate set of 31 novel interspecific yeast hybrids was developed, resulting from large-scale robot-assisted selection and breeding between six different *S. cerevisiae* and two different *S. eubayanus* strains, aiming at increasing the aromatic diversity in lager beer (Chapter 2). Many of these new hybrids produced an aromatic profile significantly different from those produced by currently available lager yeast in both lab and pilot scale fermentation tests, therefore providing a source of lager yeasts able to produce new, aromatically diverse lager beer.

1.4.6 Further improving novel generated interspecific hybrids

Newly generated interspecific hybrids often show hybrid vigour in their fermentative capacity, a broadened temperature tolerance and/or a diversified metabolite profile. Such new hybrids open new routes to address changes in the global fermented beverage market, which is characterized by a rising appreciation for low alcohol, high-flavour or unique products (56, 157).

Despite the fact that these hybrids combine interesting characteristics of their respective parental species, they can also inherit undesired phenotypes which impede their direct implementation in industrial production processes. More specifically, as non-*cerevisiae* strains are less well adapted to industrial fermentations, they often suffer from major drawbacks. Indeed, their characteristic low fermentation capacity and production of off-flavours like H_2S

and POF, are one of the many characteristics which are unwanted, but often inherited by the generated interspecific hybrid yeasts (58).

Changing industrial relevant phenotypes of novel created interspecific hybrids via traditional yeast-breeding-based strain improvement strategies is impossible due to the sexual sterility of novel generated interspecific hybrids (97, 157). Indeed, existing postzygotic barriers between members of the *Saccharomyces* clade limit interspecific hybrids to a vegetative lifestyle. Fortunately, (modern) microbiology offers a wide variety of tools, allowing to overcome or circumvent the above mentioned hybrid sterility. In the following paragraphs, three such methods are discussed in more detail, namely the generation of allotetraploid interspecific yeast hybrids, random mutagenesis and CRISPR-Cas9-based gene editing.

Generation of allotetraploid interspecific hybrids

A proposed way to overcome hybrid sterility is through the generation of allotetraploid interspecific hybrids via a rare mating of a diploid *S. cerevisiae* strain with a diploid *non-cerevisiae* yeast cell (2, 81, 147). Generated allotetraploid interspecific hybrids are in their turn able to form viable allodiploid spores, which can be used in further breeding schemes, in order to cure and/or change its industrial relevant characteristics. Since the rate of rare mating between two diploids in a population has been estimated to be between 1 out of 1 million to 100 million cells (63), different improved strategies were developed.

For example, Alexander and coworkers introduced two plasmids (pHCT2 and pHMK34 (2)), each containing a doxycycline inducible *HO* gene combined with a unique selection marker, into a diploid *S. cerevisiae* and a *S. eubayanus, S. kudriavzevi or S. uvarum* yeast strain, respectively (2). Activation of the *HO* gene potentially causes cells mother–daughter- or clone-mate- selfing, changing the mating type of the cells from a/α to a/a or α/α (Figure 9). Mixing both cultures whilst applying double drug selection (on both selection markers introduced on the two different plasmids) allows the selection of rarely formed interspecific allotetraploids with increased efficiency (0.1%). Both plasmids can be easily removed afterwards, in order to obtain marker-free synthetic interspecific allotetraploids.

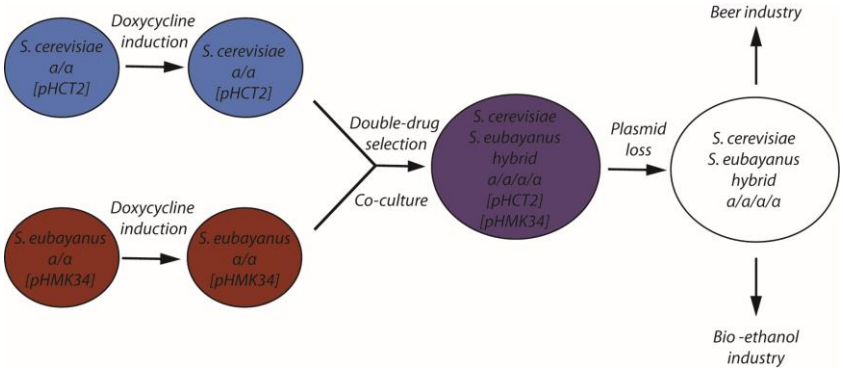

Figure 9: Generation of allotetraploid interspecific hybrids. *Induction of HO expression by a doxycycline-inducible promoter in two diploid cultures, followed by co-culture and subsequent double-drug selection, will produce hybrids at a rate approaching 1 out of 1000 cells plated. Plasmids can be cured to produce strains not containing any foreign DNA material* (2).

Another proposed strategy in overcoming the low incidence of rare mating between diploid *Saccharomyces* yeasts is the generation of auxotrophic mutants prior to rare mating. Selecting on both introduced auxotrophies increases the chance on selecting a rare mated allotetraploid significantly. Indeed, Krogerus and coworkers used this strategy to create an alloptetraploid interspecific hybrid (*S. cerevisiae* X *S. eubayanus*), which was able to sporulate and formed viable diploid spores (for breeding scheme, see Figure 10). Again, an auxotrophic mutant of the alloptetraploid interspecific hybrid was created and alodiploid spores were crossed in another rare mating round with a third, POF^- *S. cerevisiae* strain, generating another allotetraploid yeast. Spores of this allotetraploid hybrid yeast were isolated, of which some lost the ability to form POF due to meiotic segregation (81).

Although this approach allowed Krogerus and coworkers to generate a novel POF^- lager brewing yeast combining characteristics of three parental strains, the technique has some significant drawbacks. First of all, this approach depends on a rather complex breeding scheme and the generation of auxotrophic mutants of the candidate parental yeasts. More so, the dependency on two consecutive rounds of breeding implies that a significant amount of screening is needed to select segregants with wanted characteristics.

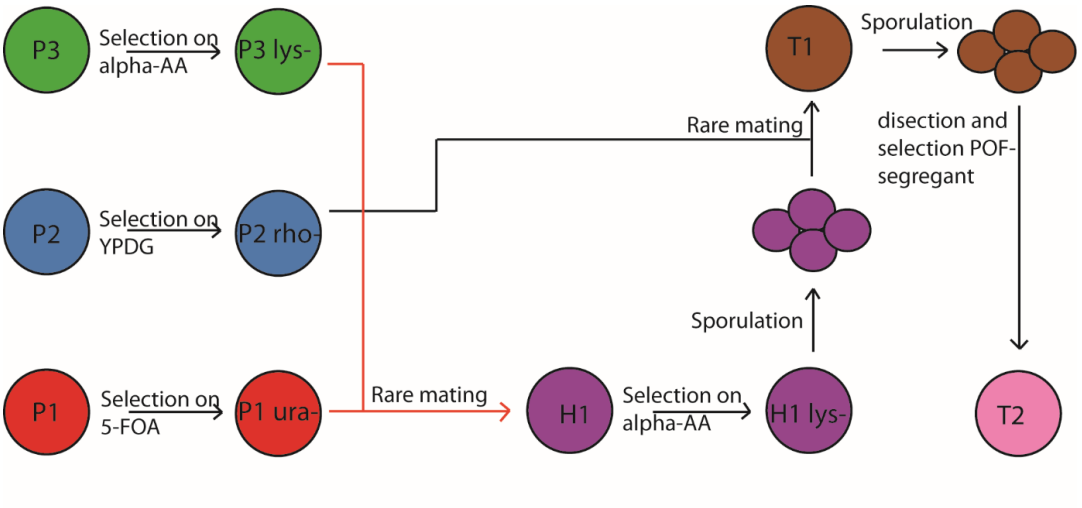

Figure 10: Improving rare mating efficiency through the introduction of auxotrophic markers. *First, mutants of parent strains P1 (S. cerevisiae 1)–P3 (S. eubayanus) carrying selection markers were selected. Next, hybrid H1 was generated via rare mating of parent strains P1 and P3. Hybrid T1, containing DNA from all three parent strains, was obtained by sporulating the allotetraploid Hybrid H1 and rare mating dissected spores with parent P2 (S. cerevisiae 2). The POF⁻ segregant Hybrid T2 was obtained by sporulating Hybrid T1 and screening spore clones for the absence of 4VG production (figure adjusted from* (81)*).*

Random mutagenesis

Second, direct modification of the non-*cerevisiae* parent to eliminate unwanted characteristics, like for example the POF phenotype, using mutagenesis has been proposed recently (36). In this study, segregants of the *S. eubayanus* strain CBS12357 were subjected to UV mutagenesis, resulting in the identification of a POF$^-$ mutant, which could be used in a breeding scheme. Because random mutagenesis is still considered being a non-GMO technique, generated mutagenized segregants and following F1 hybrids are directly applicable in industry. However, this strategy also has some limitations. Most notably the large screens required to identify positive mutants and the risk of off-target mutations with undesired phenotypic effects. And despite that a 96-well based screening method for POF production has been developed for yeast (Chapter 3; (106)), identification of a POF$^-$ mutant with no or a very limited number of mutations in other genes would require a screening setup with an even higher throughput.

CRISPR Cas9-based gene editing

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated protein (Cas9)-based genome editing technology offers a new tool to circumvent

the sterility of generated interspecific hybrids and more general of most industrial yeasts, in order to further fine tune their behaviour in industrial settings.

In general, the CRISPR-Cas9 technology in *Saccharomyces* yeasts comprises of two steps. First, the bacterial derived Cas9 endonuclease is specifically directed by a single guide (sg) RNA towards the genetic loci of interest which induces a DNA double stranded break (DSB) in the target DNA sequence (Figure 11A). More specifically, the sgRNA comprises of a CRISPR (cr) RNA and a trans-activating CRISPR (tracr) RNA, linked to each other via a linker sequence. The crRNA is a 20nt sequence, which will guide the sgRNA-Cas9 complex, via standard Watson-Crick base pairing with its complementary sequence (protospacer), towards its DNA target site. Binding specificity also depends on the presence of a specific three-nucleotide sequence flanking the three prime end of the protospacer, known as the protospacer adjacent motif (PAM; NGG sequence, with N being any possible nucleotide). The tracrRNA forms two consecutive hairpin structures, which allow complexation with the Cas9 endonuclease. When the sgRNA-Cas9 complex binds to its target site, the Cas9 nuclease domain (HNH) will cleave the DNA-strand complementary to the crRNA guide sequence, while the Cas9 RuvC-like domain will cleave the other DNA strand resulting in a DSB, situated three nucleotides upstream of the target's PAM sequence (78, 158, 180).

In a second step, the DSB is repaired via homology directed repair (HDR), which can be hijacked in order to introduce specific point mutations or to insert and delete desired sequences through recombination of the target locus with exogenously supplied DNA (=donor or repair template) (Figure 11C) (145). Within plants or other higher organisms, the preferred mechanism to repair CRISPR-cas9 induced DSBs is Non-Homologous End-Joining (NHEJ, Figure 11B), which can lead to random small insertions, deletions or point mutations.

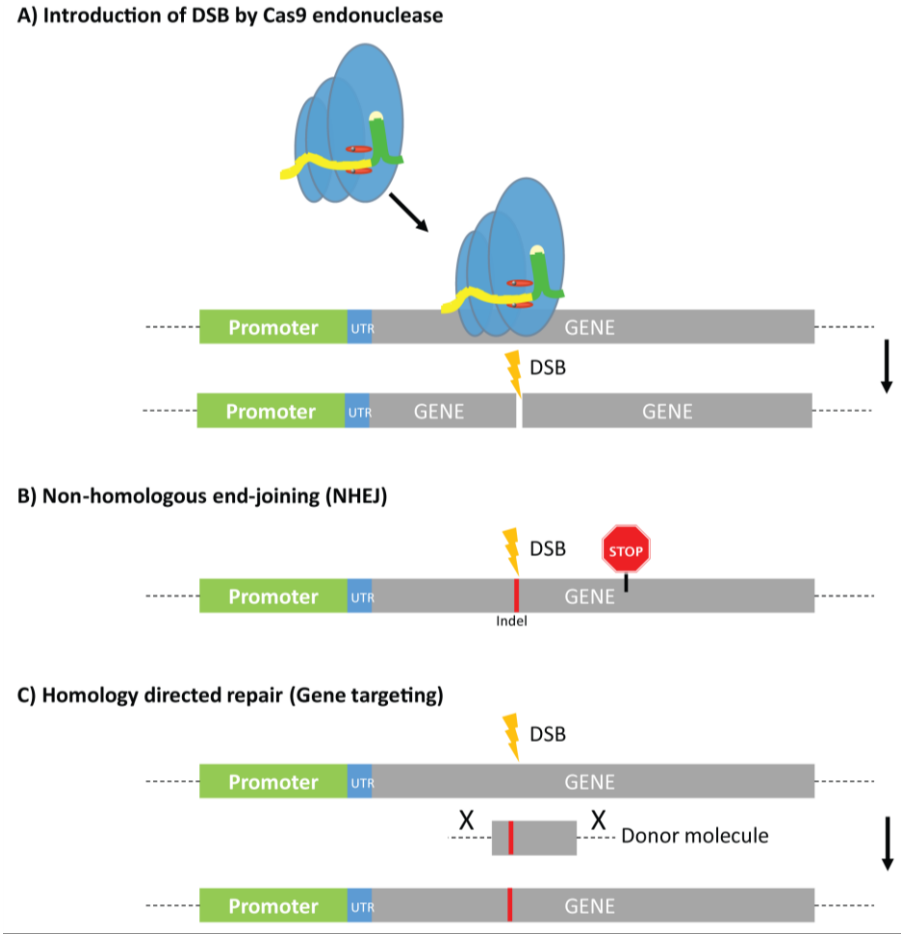

Figure 11: CRISPR cas9 in action*: A) Cas9 endonuclease, bound to a sgRNA (crRNA depicted in yellow; tracrRNA is depicted in green) will bind specifically to its matching target sequence, after which a double stranded break is introduced, located three bases upstream of the PAM sequence. B) DSB can be repaired via Non-Homologous end-joining, which can lead to small indels, deletions or point mutations. This is the prepared way in which plants will repair a cas9 introduced DSB. C) Saccharomyces yeasts will repair a Cas9 introduced DSB preferably via Homology Directed Repair, in which a repair template or donor molecule is used as a template to repair the DSB. Genetic alterations, present in the repair template, can get introduced into the gene of interest.*

In the past years, the use of CRISPR technology is prospering and has already been used to alter phenotypes of industrial *S. cerevisiae* and *S. pastorianus* yeasts (99, 145). For example, Vigentini and coworkers applied a CRISPR-based strategy to reduce the urea production of industrial wine starter yeasts (172). More recently, CRISPR Cas9-genome editing was used in yeast to introduce the production of monoterpenes as an alternative to bittering beer by the use of hops (34).

More so, de Vries and coworkers designed a new CRISPR-cas9 method, allowing them to accurately delete multi copy genes in the genetically complex *S. pastorianus* yeasts CBS1483 and Weihenstephan 34/70. Indeed, the designed strategy allowed them to simultaneously

delete the *S. eubayanus* derived *ATF1* allele, and two (Weihenstephan 34/70) or three (CBS1483) *ATF2* alleles, highlighting the multiplexing abilities of CRISPR Cas9-gene editing (174). This method was later on tested on a novel generated interspecific *S. cerevisiae* X *S. eubayanus* hybrid, but without great success (175). More precisely, the introduction of a DSB in the novel hybrid's genome caused Loss of heterozygosity instead of being repaired via HDR with the exogenous supplied repair template. Therefore, further optimization is needed to use a CRISPR Cas9-based gene editing strategy in further fine tuning novel generated interspecific hybrids (Chapter 4).

Recent favourable regulations concerning the use of CRISPR-based gene editing for organism improvement in countries like Brazil, USA, Japan and Argentina (72, 121, 179), which excludes CRISPR-engineered strains from GM labelling (For USA and Japan, a case-by-case product based decision is made, determining whether the gene edited crop or organism is regarded as being genetically modified (GM)), highlight the potential of such gene editing tools in biotechnology industries. Especially, when CRISPR-based gene editing is used to insert or alter genes originating from closely related organisms (cisgenic), the acceptance by consumers towards resulting products and the willingness to pay increases significantly (45, 179). Therefore, the application of CRISPR-engineering to introduce naturally occurring alleles could be the targeted strain improvement strategy that has been lacking for microbes used in food production. More so, the general view on other gene editing techniques like for example 'self-cloning' as being non-GMO, could potential open doors for CRISPR-based gene editing, since both techniques do not introduce heterologous obtained DNA in the gene edited variants (50, 51).

1.5 Objectives

The overall objective of this thesis is the generation of novel yeast strains in order to broaden the aromatic diversity of lager beer, crucial to reinsure the competitive position of breweries on a changing and challenging global beer market. In order to do so, artificial interspecific yeast hybrids were generated, genetically stabilized and assessed for their industrial applicability in lab scale and pilot scale brewing trials (Chapter 2).

Novel interspecific hybrids not only inherit wanted phenotypes from their respective parental species, but also tend to inherit unwanted traits. For example, one of the most important unwanted phenotypes typically inherited by interspecific hybrids from their non-*cerevisiae*

parent is the production of POF. Therefore, there is the need for good and efficient methodologies that allow further optimization of key phenotypes, like POF production, within novel and sexually sterile interspecific hybrid yeasts. Within this manuscript, we first developed a new and high-throughput screening tool, allowing to assess the POF phenotype of hundreds of different yeasts in parallel (Chapter 3). This assay was later on used when an optimized CRISPR-Cas9 based gene editing strategy was developed and applied in order to generate cisgenic POF^- interspecific hybrids, showing great potential for industrial usage (Chapter 4).

2

Generating novel interspecific hybrids in order to broaden the aromatic diversity of lager beer

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This Chapter is adjusted from:

Mertens, S., Steensels, J., Saels, V., De Rouck, G., Aerts, G., & Verstrepen, K. J. (2015). A large set of newly created interspecific yeast hybrids increases aromatic diversity in lager beers. Applied and environmental microbiology, AEM-02464.

2.1 Abstract

Lager beer is the most consumed alcoholic beverage in the world. Its production process is marked by a fermentation conducted at low (8-15°C) temperatures, and by the use of *S. pastorianus,* an interspecific hybrid between *S. cerevisiae* and the cold-tolerant *S. eubayanus*. Recent whole-genome sequencing efforts revealed that the currently available lager yeasts belong to one of only two archetypes, "Saaz" and "Frohberg". This limited genetic variation likely reflects that all lager yeasts descend from only two separate interspecific hybridization events, which may also explain the relatively limited aromatic diversity between the available lager beer yeasts, compared to for example wine and ale beer yeasts. In this chapter, 31 novel interspecific yeast hybrids were developed, resulting from large-scale robot-assisted selection and breeding between six carefully selected *S. cerevisiae* and two *S. eubayanus* strains. Interestingly, many of the resulting hybrids showed a broader temperature tolerance than their parental strains and reference *S. pastorianus* yeasts. Moreover, they combined a high fermentation capacity with a desirable aroma profile in lab scale lager beer fermentations, thereby successfully enriching the currently available lager yeast biodiversity. Pilot scale trials further confirmed the industrial potential of these hybrids and identified one strain, hybrid H29, which combines a fast fermentation, high attenuation and production of a complex, desirable fruity aroma.

2.2 Introduction

The limited genetic diversity of lager yeasts is reflected in the relative limited influence of the yeast on the aroma profile of lager beer (57), especially when compared to the immense genetic and aromatic diversity of ale *S. cerevisiae* yeast strains (155–157). While the characteristically clean, fresh flavour and aroma of lager beer is one of their most distinctive and praised traits, diversification and differentiation have become increasingly important in today's market. The development of new lager hybrids may help generating a set of distinct beers that in some ways bridge the gap between diverse, aromatic ales and fresh and highly-drinkable lagers. (57, 58, 80). Development of interspecific hybrids has proven to be a powerful approach to generate novel yeast variants with enhanced characteristics for wine making. Typically, an industrial strain of *S. cerevisiae* yeast is crossed with a wild, non-*cerevisiae* member of the *Saccharomyces sensu stricto* group, such as *S. bayanus* (100, 146–148)*, S. kudriavzevvi* (15, 131), *S. uvarum* (41, 133) or *S. mikatae* (16).

Here, we report on the development and extensive testing of 31 new lager yeast variants. Our results show that some of these novel interspecific hybrids between *S. cerevisiae* yeasts and *S. eubayanus* can outperform both of its parental strains in lager beer conditions, yielding beer with very diverse aromatic profiles, thereby enriching the potential aroma spectrum of lager beer.

2.3 Materials and Methods

2.3.1 Yeast strains used in this chapter

Parental strains for the generation of interspecific hybrid yeasts were selected from a collection of 301 industrial and wild *Saccharomyces* strains, described by Steensels and coworkers (155). Six industrial *S. cerevisiae* strains were selected based on their production of desirable aromatic compounds, sporulation capacity and spore viability (Figure 13). Additionally, two wild *S. eubayanus* strains were included as parental strains. Two different *S. pastorianus* strains, corresponding to the two types of lager yeasts (Saaz- and Frohberg type) were included in the different experiments as reference strains (Table 1) (42). An extra set of 15 different industrially used *S. pastorianus* strains were tested for their aroma and ethanol production in lab scale lager beer fermentation tests. The lab yeasts BY4742 (n), BY4743 (2n) and a confirmed tetraploid strain Y243 were included as references for Fluorescence Associated Cell Sorting (FACS) analysis.

Table 1: Saccharomyces yeast strains used in this chapter

Strain*	Species	Industry	Origin
Y134	*S. cerevisiae*	Ale beer	NA
Y184	*S. cerevisiae*	Wine	NA
Y245	*S. cerevisiae*	Ale beer	Belgium
Y397	*S. cerevisiae*	Ale beer	Belgium
Y470	*S. cerevisiae*	Ale beer	NA
Y243	*S. cerevisiae*	Bread (n = 4)	NA
BY4742	*S. cerevisiae*	Lab strain (n)	NA
BY4743	*S. cerevisiae*	Lab strain (2n)	NA
Y565	*S. eubayanus*	wild isolate	Argentina
Y567	*S. eubayanus*	wild isolate	Argentina
GSY129	*S. pastorianus*	Saaz type lager beer	Denmark
GSY131	*S. pastorianus*	Saaz type lager beer	Denmark
GSY133	*S. pastorianus*	Saaz type lager beer	NA
GSY134	*S. pastorianus*	Saaz type lager beer	Denmark
GSY137	*S. pastorianus*	Saaz type lager beer	Denmark
GSY501	*S. pastorianus*	Saaz type lager beer (ref)	NA
GSY509	*S. pastorianus*	Saaz type lager beer	NA
GSY132	*S. pastorianus*	Frohberg type lager beer (ref)	The Netherlands
GSY135	*S. pastorianus*	Frohberg type lager beer	Canada
GSY515	*S. pastorianus*	Frohberg type lager beer	The Netherlands
GSY516	*S. pastorianus*	Frohberg type lager beer	The Netherlands
Y5	*S. pastorianus*	Frohberg type lager beer	Belgium
Y447	*S. pastorianus*	Frohberg type lager beer	Germany
Y449	*S. pastorianus*	Frohberg type lager beer	Germany
Y453	*S. pastorianus*	Frohberg type lager beer	Germany
Y454	*S. pastorianus*	Frohberg type lager beer	Germany
Y473	*S. pastorianus*	Frohberg type lager beer	Czech republic

* Strains denoted with a "Y" code are derived from the yeast collection stored at KU Leuven University. "GSY" strain codes are the codes used in the previous research of Dunn and Sherlock (42).

2.3.2 Sporulation and tetrad dissection of possible parental strains

Sporulation of selected parental strains was induced on acetate medium (potassium acetate 1% w.v^{-1}, amino acid mix 0.05% w.v^{-1}, and agar 2% w.v^{-1}) after five to ten days at 25°C. Subsequently, sporulation capacity was assessed using a light microscope (40x). The ascus wall was digested with 4 mg.ml^{-1} zymolyase (Seikagaku, Japan) suspension (dissolved in 2 M sorbitol), incubated for 3 minutes at room temperature. Tetrads were dissected using a micromanipulator (MSM-singer instruments) on YPD-agar (bacto peptone 2% w.v^{-1}, yeast extract 1% w.v^{-1}, glucose 2% w.v^{-1}, agar 2% w.v^{-1}).

2.3.3 Hybrid generation through spore-to-spore mating and confirmation of hybrid nature

Sporulation was induced as described previously (152, 155). Spores were isolated *en masse* as described by Snoek and coworkers (152) and stored at -80°C in glycerol yeast peptone dextrose (GYPD) medium (2% wv^{-1} bacto peptone, 1% $w.v^{-1}$ yeast extract, 2% $w.v^{-1}$ glucose and 25% $w.v^{-1}$ glycerol).

Hybridization was induced by placing single spores from both parental strains together with a micromanipulator (MSM-singer instruments) on YPD-agar (2% wv^{-1} bacto peptone, 1% $w.v^{-1}$ yeast extract, 2% $w.v^{-1}$ glucose and 1.5% $w.v^{-1}$ agar) followed by visual inspection of zygote formation after 6-8 hours of incubation at room temperature (Figure 12). Candidate interspecific hybrids were purified by streaking on synthetic 12 °P malt agar medium (12% $w.v^{-1}$ synthetic malt extract (8EBC Brouwland, Belgium) and 1.5% $w.v^{-1}$ agar). Hybrids were confirmed through a species multiplex PCR (see below). PCR-confirmed interspecific hybrids were streaked another three consecutive times on 12°P wort medium prior to long term storage at -80°C to ensure strain purity.

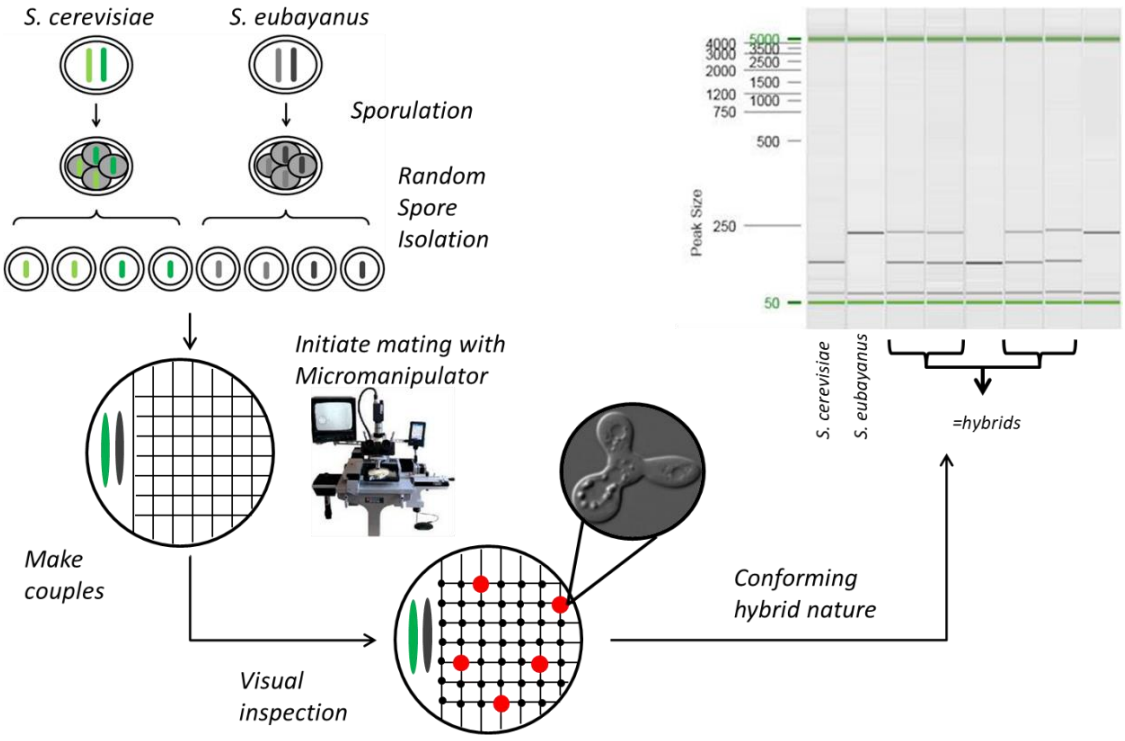

Figure 12: Schematic overview interspecific hybrid generation. *Strains of both selected parental species were sporulated and single spore suspensions were generated. Using a micromanipulator, single spores of both parental strains were placed together on a agar plate. After incubation at room temperature, possible zygote formation was assessed and confirmed via a species multiplex PCR2.3.3.*

2.3.4 Species multiplex PCR

Two primer pairs were used for the species multiplex PCR, each targeting a specific part of one of the parental species obtained *FAL1* genes (112, 130). Primers Scer F2 (5'-GCG CTT TAC ATT CAG ATC CCG AG-3') and Scer R2 (5'-TAA GTT GGT TGT CAG CAA GAT TG-3') amplify an 150 bp amplicon of the *S. cerevisiae* genome. Primers Seub F3 (5'-GTC CCT GTA CCA ATT TAA TAT TGC GC-3') and Seub R2 (5'-TTT CAC ATC TCT TAG TCT TTT CCA GAC G-3') generate a 228bp *S. eubayanus* specific amplicon (Figure 14). PCR conditions were: 3 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds of 72°C, followed by a last cycle of 5 minutes at 72°C and subsequent cooling to room temperature (RT). Candidate hybrids showing two bands were considered to be interspecific hybrids (Figure 14).

2.3.5 Amplified inter δ-sequence DNA polymorphism analysis

Primers delta12 (5'-TCA ACA ATG GAA TCC CAA C-3') and delta21 (5'-CAT CTT AAC ACC GTA TAT GA-3') were used, as previously described by Legras and karst (Figure 14) (86).

2.3.6 Random Amplified polymorphic DNA (RAPD) analysis

RAPD-analysis was carried out by using the R3-primer (5'-ATG CAG CCA C-3'), as described previously (32, 131), using the following PCR protocol: 4 minutes at 94°C, 35 cycles of 25 seconds at 94°C, 30 seconds at 42°C and 1.30 minute of 72°C, followed by a last cycle of 5 minutes at 72°C and subsequent cooling to RT (Figure 14).

2.3.7 Flow cytometry

Cells were grown overnight to stationary phase in 1mL YPD medium at RT on a shaker set at 300 rpm. Next, cells were washed in 1 mL deionized water and resuspended in 1 mL 70 % $v.v^{-1}$ ethanol and incubated at 4°C for at least 16 hours while rotating. The cultures were washed once with 500 µL SC-buffer (50mM trisodiumcitrate.dihydrate; pH 7.4), resuspended in 1 mL SC-buffer containing 0.25$mg.mL^{-1}$ RNAse A (Thermo Scientific), and incubated for one hour at 50°C, after which 50 µL Proteinase K solution (20 $mg.ml^{-1}$, Fisher Bioreagents) was added, followed by an additional incubation of one hour at 50°C. Cell suspensions were washed once with 500 µL SC-buffer and resuspended in 1 mL SC-buffer. Propidium iodide (Sigma-Aldrich, Belgium) was added to a final concentration of 16 $µg.mL^{-1}$ and cells were incubated overnight at 4°C while rotating to stain the DNA content of the cells. Finally, the cells were washed once with SC-buffer and the DNA content of single yeast cells was analysed using a Fluorescent

Associated Cell Sorting System (FACS, BD Biosciences, Belgium). Analysis of the obtained FACS data, based on Mixed Gaussian Models, was performed in R (163).

2.3.8 Genetic stabilization of generated hybrids

Obtained interspecific hybrids were subjected to a high throughput genetic stabilization protocol. Each hybrid was individually inoculated into 750 µL industrial-grade high glucose containing 12°P wort medium (provided by a Belgian brewery) in a 96 deep-well plate and incubated statically at 16°C for six days. At this stage, cultures were ten times diluted in 742.5 µL of fresh wort medium. This incubation/dilution procedure was repeated 12 times, where after genetic stability of the hybrids was assessed by genetic finger printing PCR's of four biological replicates (inter δ-sequence DNA polymorphism analysis and R3-RAPD for four single colony isolates, described above; and Figure 14) (131).

2.3.9 Lab scale lager fermentations

The fermentation protocol was designed to mimic industrial lager fermentations. First, yeast was propagated by inoculation in 5 mL 4% yeast peptone maltose (YPM; 2% $w.v^{-1}$ bacto peptone, 1% $w.v^{-1}$ yeast extract and 4% $w.v^{-1}$ maltose) medium at RT and 300 rpm. After 16 h incubation, 1 mL of the culture was transferred to 50 mL YPM (4%) medium in a 250mL Erlenmeyer flask and incubated at 20°C and 200 rpm for 16h. After this second propagation, optical density was measured at 600nm (OD_{600}) and the pregrowth culture was used to inoculate 150 mL of industrial grade wort medium (high glucose containing 12°P wort, sparged with pressurized air for one hour to saturation, provided by a Belgian brewery) to a starting OD_{600} of 0.3 (approximately $2.1*10^7$ $cells.mL^{-1}$). The 250 mL bottles were equipped with Ankom system Gas monitors for online measurement of gas production (Ankom, USA). The headspace was flushed with nitrogen gas prior to incubation at 16°C. During fermentation, a constant overpressure of 0.5 bar (to atmospheric pressure) was applied. CO_2 production was monitored in real time for all fermentations with the Ankom systems, and fermentations were stopped when all strains stopped fermenting (after 13 days). Next, the fermentations were cooled on ice to prevent evaporation of the volatile compounds, and samples for chromatographic analysis and ethanol measurements were taken. The leftover fermented medium was used for sensory analysis.

2.3.10 Temperature tolerance assay

Yeasts were propagated in 200 µl YPD (2%) for 16h at 30°C (shaking at 900 rpm). Next, the yeast cultures were diluted to OD_{600} = 1, 0.1, 0.01 and 0.001 in isotonic phosphate saline buffer. Subsequently, 5 µL of the dilution series was spotted in biological duplicates on YPD agar plates using the Rotor HDA (Singer Instruments, UK), and incubated at eight different temperatures until sufficient growth could be observed: 4°C (15 days), 8°C (5 days), 10°C (5 days), 16°C (2 days), 25°C (2 days), 30°C (2 days), 37°C (2 days) and 41°C (2 days). Plates were scanned and colony size was quantified using the Screenmill software in ImageJ, as described in (37). Colony sizes at different temperatures are represented as z-scores (calculated per strain and per temperature).

2.3.11 Determination of 4-vinyl guaiacol production capacity

Yeasts were grown for 48 h at 30°C in 5 mL 2% YPD to which 0.1mg.mL^{-1} Ferulic Acid (Sigma-Aldrich, Belgium) was added. Production of 4-vinyl guaiacol (4VG) was measured using Head Space-Gas Chromatography with Flame Ionization Detector (HS-GC-FID).

2.3.12 Pilot scale fermentation tests

A commercial Belgian Pilsner Malt was used to produce wort in the 5 hl pilot brewery of KU Leuven, technology campus Gent. 87 kilograms of fine milled Pilsner malt (wet disc milling, Meura) were mixed with 1.91 hL de-aerated reversed osmosis brewing water containing 80 mg.L^{-1} $CaCl_2$ and 30% v.v^{-1} lactic acid (precise volume to be added is malt dependent). Mashing-in occurred at 64°C and pH 5.3. The following brewing scheme was used: 64°C (30 min), 72°C (20 min), 78°C (1 min) (rise in temperature at 1°C.min^{-1}), after which the wort was filtered through a membrane assisted thin bed filter (Meura 2001) and sparged with 2.5 L.kg^{-1} water (extract of last runnings 1.5°P and 1°P after final compression). The extract of the combined sweet wort was 14.5°P. The sweet wort was mixed with brewing water at the onset of boiling to obtain an extract content of 11.5°P. Wort boiling was conducted for 60 min at atmospheric boiling in a boiling kettle with internal boiler (~5% evaporation). At the end of boiling, 0.2 mg.L^{-1} Zn^{2+} ions were added, as well as 3.85 g.hL^{-1} iso- α -acids extract, aiming at 25 mg.L^{-1} iso- α -acids in the finished beer (utilization ~ 65%). The wort was clarified in an open whirlpool as follows: filling in for 6 min, rest of 20 min, emptying in 20 min at 95°C. After cooling and aeration, the wort (12°P) was divided in 10x 50L fermenters and pitched with 10^7

cells.mL^{-1} of the appropriate yeast strain. Starters were propagated at 25°C in 152.5 g.L^{-1} wort extract (Brouwland; Belgium) until the cell titre was high enough for pitching. The duration of the primary fermentation at 12°C in cylindroconical tanks was strain dependent: fermentations were stopped if a minimal apparent degree of fermentation (ADF) of 72% was achieved or if the respective yeast stopped fermenting within three weeks after pitching. Fermentations that took longer than three weeks were omitted from further analysis. Green beer was matured for 10 days at -0.5°C in 50L beer kegs. The final beer was filtered using a cellulose sheet filter system (pore size 1µm) prior to CO_2 saturation up to 5.6 g.L^{-1} and packaging with a 6 head rotating counter pressure filler (monobloc, CIMEC, Italy) using double pre-evacuation with intermediate CO_2 rinsing and overfoaming with hot water injection before capping (final oxygen levels: below 50 ppb). Finished beers were sampled for chromatographic analysis and ethanol measurements. Also, a professional tasting panel assessed the different finished beers for their aroma, flavour, taste/mouthfeel and overall impression.

2.3.13 Data analysis and data visualization

To correct for noise, obtained head space gas chromatography Flame Ionization Detector (HS-GC-FID)- and temperature tolerance data were converted to Z- scores as follows:

$$Z\ score = \frac{(X - \mu)}{\sigma}$$

With:

X = concentration measurement or colony size

µ = mean value of all strains per measured component or temperature (column-zscores) or mean colony growth per strain at the different temperatures (row-zscores)

σ = standard deviation of values per tested aroma compound or per tested temperature of all strains or standard deviation per strain at the different temperatures (row-zscores)

BioNumerics (Applied Maths, Belgium) was used to analyse and cluster the strains based on their phenotypes. A similarity matrix was build based on Euclidean distances and an Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm was used for clustering.

The temperature tolerance data and aroma production in the lab scale fermentation tests were statistically assessed using a Kruskal-Wallis one-way analysis of variance test (82) in combination with posthoc Dunn tests with False Discovery Rate (FDR) corrected P-values, based on the Benjamini-Hochberg correction (17, 43). Statistical tests were performed in R (163).

2.4 Results

To expand the genetic and phenotypic diversity of lager yeasts, we generated 31 new interspecific yeast hybrids between *S. cerevisiae* and *S. eubayanus*. These hybrids were assessed for their temperature tolerance and their fermentation capacity and aroma production in lab-scale fermentations. Subsequently, the fermentation performance of four selected hybrids was tested in a pilot scale brewery.

2.4.1 Selection of parental strains for the interspecific hybrids

Careful selection of optimal parental strains is vital for successful breeding experiments. Previously, our research group screened 301 industrial and wild strains from a large *Saccharomyces* yeast collection for different industrially relevant traits (115, 152, 155). Based on these data, six *S. cerevisiae* strains were selected using four selection criteria: production of a diverse aroma profile, high sporulation capacity, high spore viability and efficient maltose fermentation (Figure 13). Strains Y565 and Y567 were chosen as *S. eubayanus* parental strains, since they were able to generate viable spores and also showed high tolerance towards cold temperatures (Figure 15).

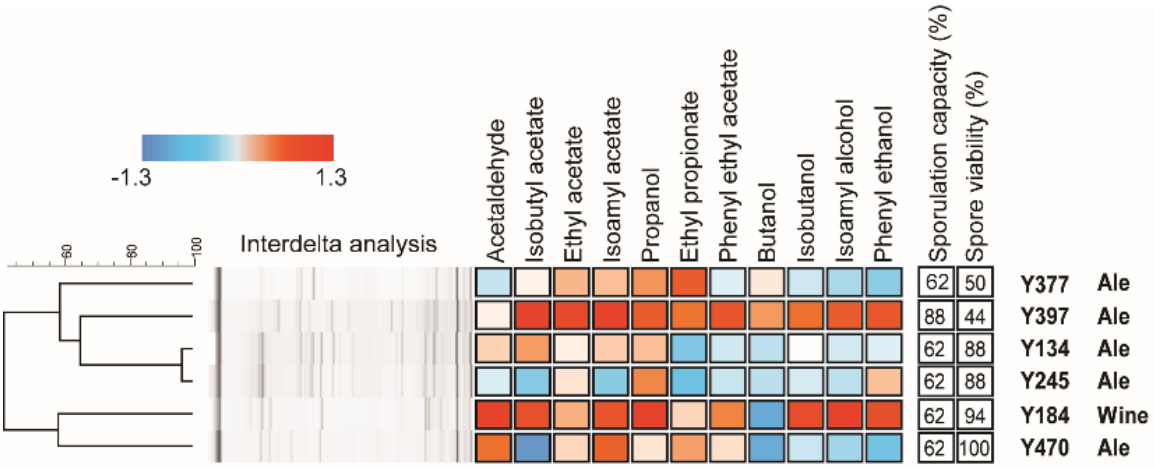

Figure 13: Main characteristics of selected S. cerevisiae parental strains*. Genetic relatedness was determined by interdelta DNA fingerprinting, and subsequent clustering was performed using Bionumerics software. Sporulation capacity and spore viability are represented as percentages, whereas aroma production is visualized relatively to a representative set of 104 ale beer strains (represented as Z-scores), previously screened by Steensels et. al. (2014). White: the same as average. Blue: lower than average. Red: higher than average.*

2.4.2 Development of interspecific yeast hybrids using spore-to-spore mating

Previous studies indicate that the success rate of interspecific mass mating experiments is very low, likely because of the prezygotic barrier between species from the *Saccharomyces* genus (80, 131). To overcome this limitation, spore-to-spore mating was used to cross yeast of different species (156). However, even this approach proved to be relatively inefficient, with 2061 mating attempts to yield a total of 31 different interspecific hybrids (overall hybridization yield of 1.5%, Table 2; hybrids were confirmed through the developed species multiplex PCR; see paragraph 2.3 material and methods and Figure 14). Moreover, mating efficiency seemed to depend on the parental strains: while mating Y134 with Y567 resulted in a hybridization yield of 3.85%, none of the 135 attempts to cross Y377 with Y565 yielded viable hybrids.

Table 2: Overview developed interspecific hybrid yeasts

	Y565		Y567	
	# PCR- confirmed hybrids	**Hybridization success rate (%)**	**# PCR- confirmed hybrids**	**Hybridization success rate (%)**
Allodiploid				
Y470	3 (**H1-3**)	2.34	3 (**H4-6**)	1.17
Y184	3 (**H7-9**)	2.56	2 (**H10-11**)	3.33
Allotriploid				
Y134	3 (**H12-14**)	1.75	3 (**H15-17**)	3.85
Y245	3 (**H18-20**)	2.22	3 (**H21-23**)	1.93
Y377	0	0	3 (**H24-26**)	1.39
Y397	2 (**H27-28**)	1.23	3 (**H29-31**)	2.47

It is known that newly generated interspecific hybrids may show temporary genome instability, with several chromosomal rearrangements taking place in the first cell divisions after the hybridization event. Prior to phenotypic characterization, the genomes of the newly developed hybrids were therefore stabilized by growing interspecific hybrids for approximately 70 generations in industrial lager beer medium (see paragraph 2.3 Materials and Methods for details). This number is shown to be sufficient to stabilize the genome of newly formed interspecific yeast hybrids within the *Saccharomyces* genus (16, 41, 131, 133). Genetic stability was confirmed by genetic fingerprinting and hybrids were considered stable when, after stabilization, both the obtained interdelta and R3-RAPD band patterns were identical for four tested biological replicates (Figure 14).

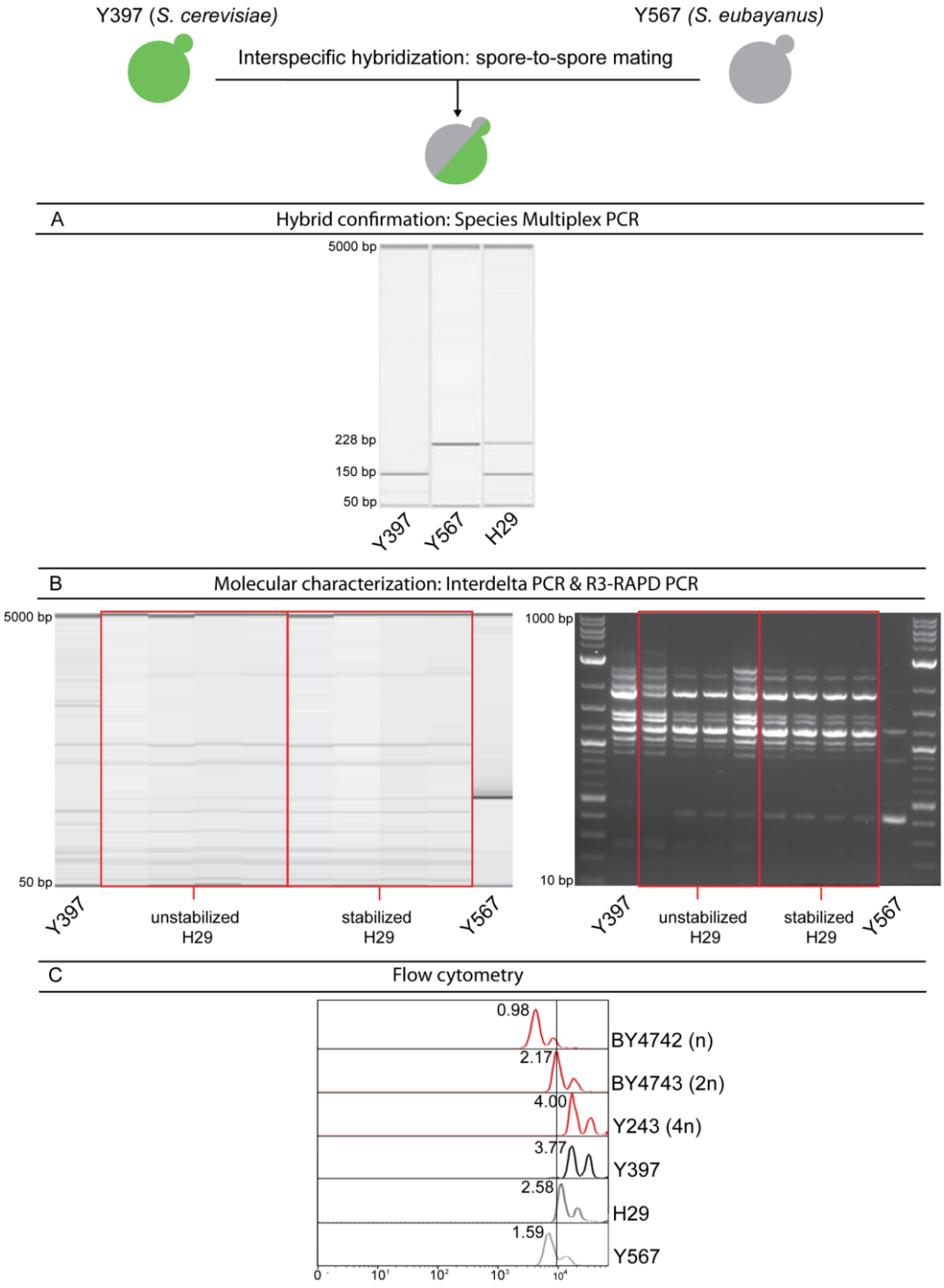

Figure 14: Example of performed molecular analyses on hybrid nature and stability check of hybrid H29 after 70 generations. *(A) Hybrid nature of possible hybrids was assessed based on the species multiplex PCR. Two bands (each originating from both parents), confirm the hybrid nature of H29 (ale-type S. cerevisiae Y397 X S. eubayanus Y567). (B) Hybrid genome stability was assessed performing two DNA-fingerprints before (lanes 2-5) and after the applied stabilization protocol (lanes 6-9) and compared with its respective parental strains (interdelta analysis (left) and RAPD-R3 (right)). Interdelta profiles of hybrids before and after stabilization were the same. RAPD-R3 fingerprints of hybrid H29 before stabilization showed differences, whereas after the stabilization protocol no heterogeneity in hybrids was detected. This indicates that the newly developed hybrids after stabilization were stable. (C) PI staining result H29 and its corresponding parental strains Y397 and Y567.*

2.4.3 Propidium iodide staining reveals ploidy differences within generated interspecific hybrids

Propidium iodide staining and flow cytometry revealed ploidy differences between the selected *S. cerevisiae* parental strains, which in turn led to ploidy differences within the developed interspecific hybrids (Table 2 and Table S1). *S. cerevisiae* strains Y470 and Y184 showed a DNA content of respectively 1.55 and 1.77, indicating that these strains are likely aneuploid, but are probably derived from a diploid strain. Therefore, mating the haploid segregants of these strains with haploid segregants from *S. eubayanus* resulted in allodiploid hybrids. However, the remaining four other parental *S. cerevisiae* strains harboured a larger genome (>2n). Strain Y134 was (allo)triploid, whereas the genome size of Y245, Y377 and Y397 was similar to the genome size of the tetraploid reference strain (Table S1). As expected, hybridization of (diploid) segregants of these strains with haploid *S. eubayanus* segregants yielded hybrids with an allotriploid genome. Interestingly, differences in genome size were observed between hybrids originating from the same parental strains. For example, the genome of H27 was 1.44 times larger than the genome of hybrid H28, even though both hybrids were the result of crossing the same two parental yeast strains Y397 and Y565 (Table 2 and Table S1). This phenomenon indicates that segregants from the same alloploid *S. cerevisiae* parent can differ in ploidy, or that the genomes of interspecific hybrids originating from the same parents can stabilize differently, resulting in different genomic configurations, as reported in previous studies targeting interspecific hybrids (16, 41, 83, 111).

2.4.4 Newly developed interspecific hybrids show a broad temperature tolerance range

The growth capacity of the developed hybrids was assessed at a wide range of different temperatures (4°C, 8°C, 10°C, 16°C, 25°C, 30°C, 37°C and 41°C) and compared to the growth of their parental strains and two reference *S. pastorianus* strains (one Frohberg type (GSY132) and one Saaz type (GSY501)). These data revealed that temperature tolerance was species-specific, with *S. cerevisiae* showing optimal growth at high temperatures (37°C and 41°C), while *S. eubayanus* (and the *S. pastorianus* reference strains) showed good performance at low temperatures, but proved unable to grow at 37°C (Figure 15).

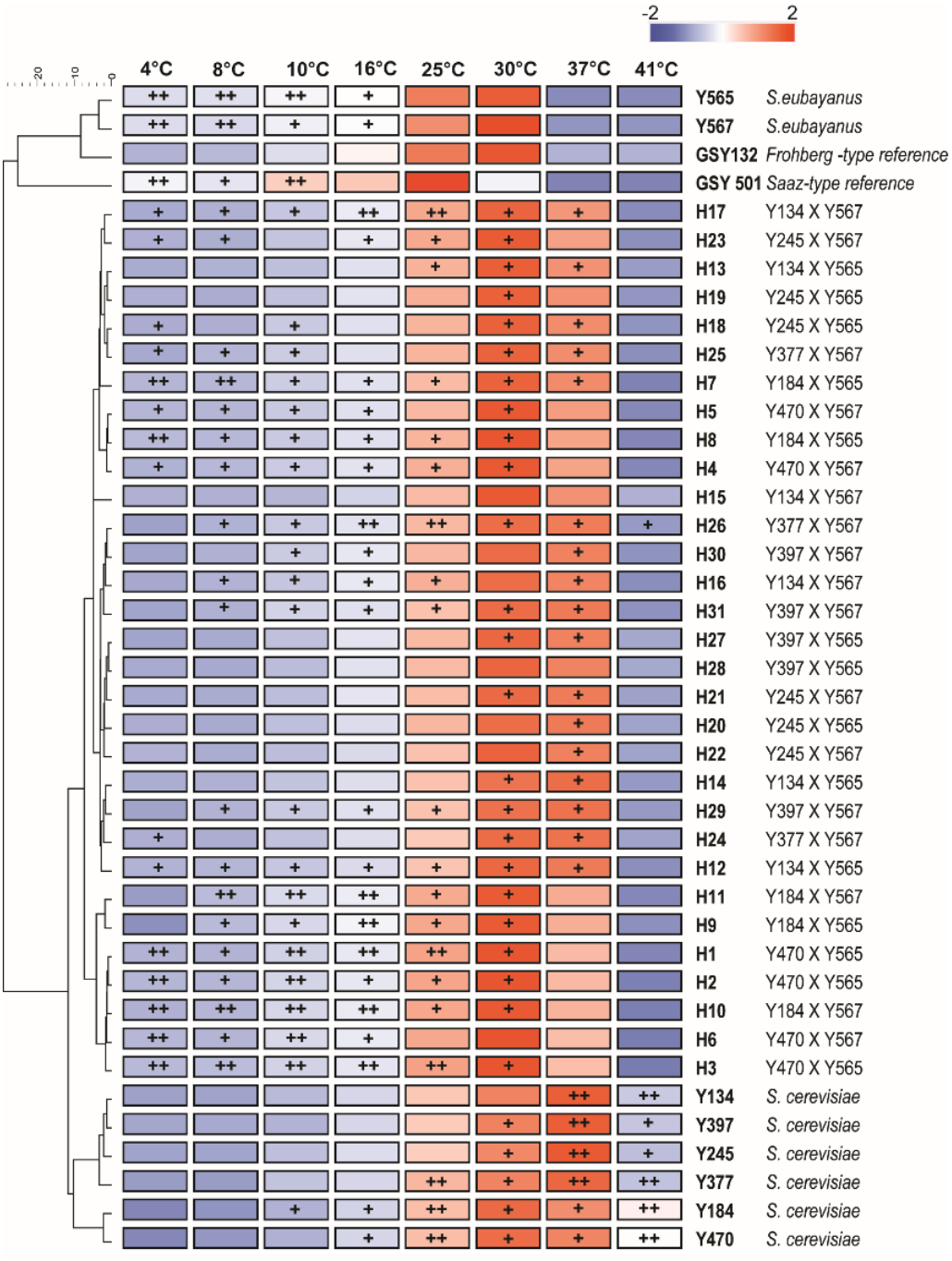

Figure 15: Relative growth speed of the parental strains, the newly generated hybrids and two commercial lager yeasts at different temperatures *(4°C, 8°C, 10°C, 16°C, 30°C, 37°C and 41°C). Growth rates were calculated as Z-scores. Pair wise similarities were calculated by Euclidean distance, and a UPGMA clustering algorithm was applied to cluster the data. Colours represent the calculated Z-scores per strain, with blue indicating a lower-than-average growth speed, white indicating average growth and red indicating higher-than-average growth (calculated over the rows). Additionally, plus signs indicate whether a particular strain has a better growth capacity than the average growth capacity of all tested strains at a particular temperature (z-score calculated over the columns, between zero and one (+) or above one (++)). Note that some hybrids share the relatively high growth rate at lower temperatures of S. eubayanus with the capacity of S. cerevisiae to grow at temperatures of 37°C and higher. Relative growth rates of all tested strains is represented in Table S15.*

At 4°C, almost all interspecific hybrids (except H9, H15 and H28, which were not able to grow at this low temperature) showed a higher growth capacity compared to their corresponding *S. cerevisiae* parental strain (only *S. cerevisiae* Y184 wine strain showed some growth at this temperature). The *S. eubayanus* parental strains and the Saaz type reference *S. pastorianus* strain showed relatively good growth at this low temperature, confirming their cold-tolerant nature.

The growth capacity of the newly generated interspecific hybrids at 37°C resembled the growth capacity of their corresponding *S. cerevisiae* parental strains, while the reference *S. pastorianus* strains and the *S. eubayanus* parental strains were not able to grow in this condition. At 41°C, only the selected parental *S. cerevisiae* strains and hybrids H26 and H27 were able to grow.

Overall, the generated hybrids showed a significantly higher growth capacity at low temperatures (4°C, 8°C, 10°C and 16°C) than the selected *S. cerevisiae* parents and a higher growth capacity at high temperatures (30°C and 37°C) than both *S. eubayanus* parents (Dunn test with Benjamini Hochberg corrected P- values < 0.05; Table S2) These results confirm that interspecific mating between *S. cerevisiae* and *S. eubayanus* yeasts can generate hybrids that show a broad temperature tolerance, equipping them with a competitive advantage at low temperatures compared to the corresponding *S. cerevisiae* parental strain.

2.4.5 Interspecific hybrid yeasts outperform their parental strains in terms of fermentation capacity in lager fermentations

Next, we assessed the potential of the new interspecific hybrids to produce aromatic lager beer. All 31 interspecific hybrids and their respective parental strains were tested in parallel lab-scale lager beer fermentations (see paragraph 2.3 Materials and Methods for details). Seven commercial Saaz- and ten Frohberg- type *S. pastorianus* strains were included as a reference.

To determine whether the strains had completed the beer fermentation, ethanol concentrations were measured at the end of fermentation. Ten out of 11 developed allodiploid (90.9%) and 15 out of 20 allotriploid interspecific hybrids (75.0%) were able to produce more ethanol than both their parental strains in a thirteen-day static fermentation process (Figure 16 and Table S1). On average, hybrids showed 28.8% higher ethanol

production than their corresponding best performing parental strain, suggesting that the interspecific hybrids outperform their parental strains in terms of fermentation capacity in lager beer fermentation conditions (16).

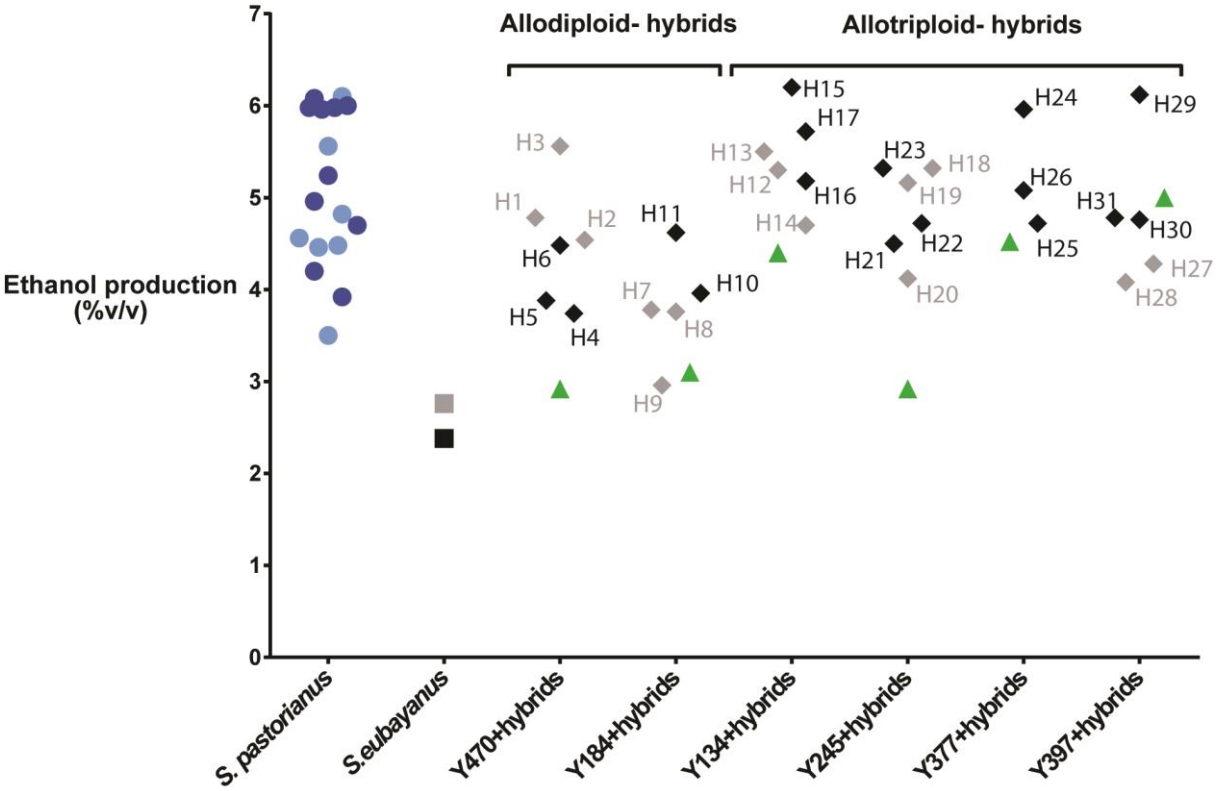

Figure 16: Ethanol production in lab scale lager fermentations. *The figure depicts the %(v/v) ethanol produced by the different hybrids (black or grey diamonds; color corresponds to corresponding S. eubayanus parental strain), together with their corresponding S. cerevisiae parental strain (green triangles), both S. eubayanus parental strains (Y565: grey square and Y567: black square) and 7 Saaz-type (light blue circles) and 10 Frohberg- type (dark blue circles) reference S. pastorianus yeasts.*

Moreover, the ethanol production by many of the new interspecific hybrids is similar to the concentrations obtained with the commercial reference *S. pastorianus* strains. Interestingly, three hybrids, H15, H29 and H27 (all hybrids from different *S. cerevisiae* parents crossed with Y567) showed a similar ethanol production capacity compared to the best reference *S. pastorianus* strains. Of these, H15 (Y134 X Y567) produced the highest final ethanol concentration (6.20% vv^{-1}, Table S1).

2.4.6 Interspecific hybrid yeasts show diversified aroma production in lager beer fermentations

Both *S. eubayanus* parental strains produced a similar aroma profile, characterized by a relatively modest production of acetate- and ethyl esters, and higher concentrations of fusel alcohols. The latter are often described as having alcoholic and solvent-like aromas, and are generally considered as unpleasant in beer when present in high concentrations (65, 104). Additionally, sensorial analysis revealed the presence of strong sulphur-like off-flavours in both *S. eubayanus* fermentations, which might be due to the fact that the beer was not maturated (lagered) (Table S1). Interestingly, the pronounced aromatic diversity of the selected *S. cerevisiae* parental strains in ale fermentations is largely reduced when these strains are applied in lager beer fermentations, highlighting the strong influence of environmental parameters (medium composition, temperature and agitation) on yeast aroma production. For instance, strain Y184 produced similar concentrations of aroma compounds than the *S. eubayanus* parental strains, whereas this yeast strain was one of the most aromatic strains in ale beer fermentations (Figure 13). Strains Y134 and Y470 were characterized by an overall low aroma production. Strain Y245 (which, like Y184, showed a relative low fermentation capacity) produced higher amounts of acetate- and ethyl esters, and lower amounts of undesirable higher alcohols, resulting in an overall fruity and pleasant beer. The aroma profile of Y377 highly resembled that of strain Y245, except for the production of isoamyl alcohol. Y397 was the only parental *S. cerevisiae* strain that was able to combine a good fermentation capacity with an overall high production of aroma compounds in these small scale lager beer fermentations.

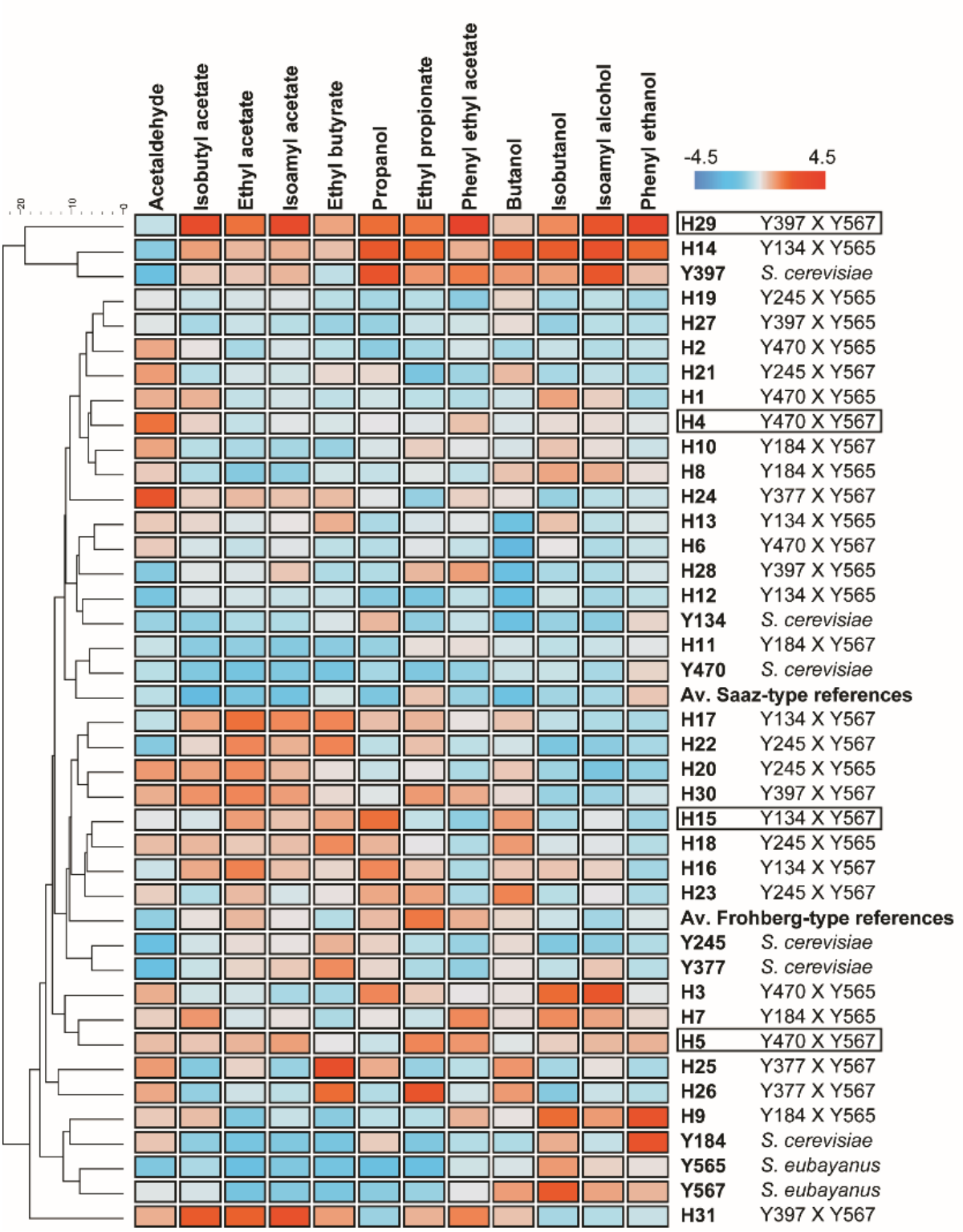

Figure 17: Visual representation of the aroma production of generated hybrids, together with their corresponding parental strains and average values of 7 Saaz type and 10 Frohberg type S. pastorianus strains. *Colours represent the calculated Z-scores (calculated over the columns), with blue indicating a lower-than-average production, white indicating average production and red indicating higher-than-average production of the measured aroma compound in question. Pairwise similarities were calculated by Euclidean distance, and a UPGMA clustering algorithm was applied to cluster the data. Selected hybrids for further pilot scale fermentation experiments are highlighted.*

Interestingly, the newly developed interspecific hybrids produced widely diverse aroma profiles. For example strain H9 (Y184 X Y565) produced a similar aroma profile than the *S. eubayanus* parental strains, whereas other hybrids, like H31 (Y397 X Y567), produced higher concentrations of esters and lower concentrations of higher alcohols reminiscent of some *S. cerevisiae* strains used in ale fermentations. Strain H29 (also Y397 X Y567) was not only one of the hybrids with the best fermentation capacity, but was also characterized by a high production of the quantified aromatic compounds. Interestingly, hybrids from the same parents produced different aromas. For example, strain H14 turned out to be one of the most aromatic hybrids, whereas hybrid H12, which shares its parental strains with strain H14 (Y134 X Y565), showed an overall low aroma production.

In general, the developed hybrids produced significantly more isoamyl acetate (IA), a fruity aroma compound, compared to their respective parental strains. IA levels were increased in 21 of the 31 developed hybrids (10/11 allodiploid and 11/21 allotriploid), resulting in lager beer with distinctive fruity banana and pineapple notes (Table S1). Moreover, isoamyl acetate concentrations obtained with some of the newly generated hybrids were also markedly higher than those obtained with the 17 reference *S. pastorianus* strains (Figure 18; Mann-Whitney U test; $p< 0.05$). Strain H29 showed the highest IA production (2.66 $mg.L^{-1}$) which was 5.11 and 2.11 times more IA than the average of the Saaz type- and Frohberg type reference *S. pastorianus* strains, respectively. Besides its high IA production, the production of ethyl acetate, which confers a solvent- like flavour, was only slightly increased compared to its corresponding parents, and still below its reported flavour threshold in lager beer (30 $mg.L^{-1}$) (104). Since this strain also showed a good fermentation capacity, it was earmarked as an interesting candidate for the production of highly aromatic lager beer.

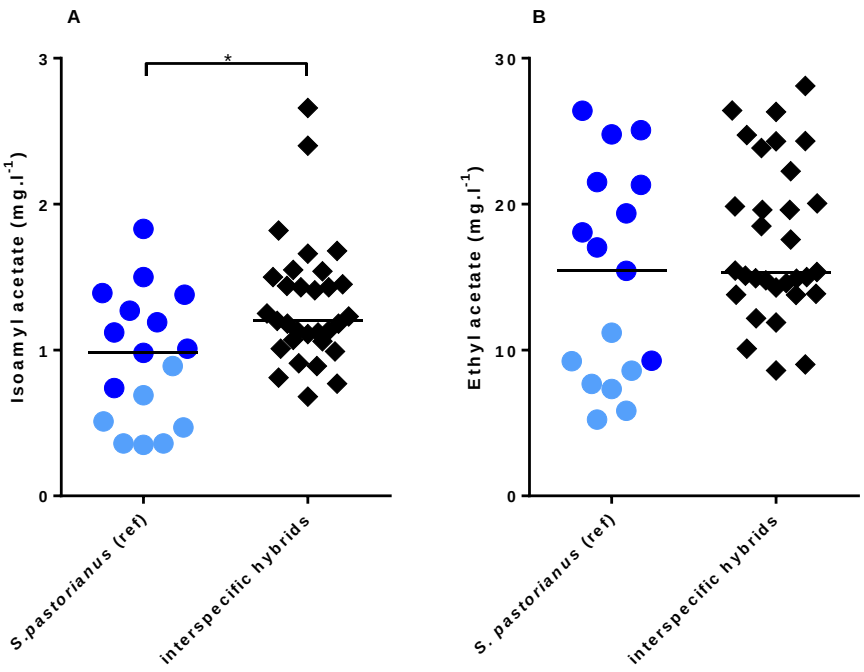

Figure 18: Comparison of (A) isoamyl acetate and (B) ethyl acetate production of developed interspecific hybrids and 17 reference S. pastorianus strains. *The production of isoamyl acetate and ethyl acetate for 7 Saaz type (light blue circles) and 10 Frohberg type (dark blue circles) S. pastorianus strains is compared to the production of the developed interspecific hybrids. The median aroma production is depicted as a black horizontal line. The developed interspecific hybrids produced a significant higher amount of isoamyl acetate compared to the 17 S. pastorianus strains (Mann-Whitney test; p< 0.05 (*))*

Principal Component Analysis (PCA) of all measured aroma compounds further reveals how the novel interspecific hybrids increase the aromatic diversity of lager beer altogether (Figure 19). Plotting principal component 1 (PC1) versus PC2, reveals a clear separation in the aroma profiles of both types of reference *S. pastorianus* strains, with the Saaz type reference strains showing a narrower aromatic diversity. Interestingly, the aromatic diversity of the new interspecific hybrids covers and expands the aromatic diversity of the more aromatic Frohberg type reference *S. pastorianus* strains. Six hybrids (H3, H9, H14, H17, H29 and H31) were characterized by an aroma profile clearly differentiating from both types of reference *S. pastorianus* strains. More interestingly, Plotting PC1 versus PC3 and PC2 versus PC3 revealed a clear difference in aroma production between the interspecific hybrids and the reference Frohberg type *S. pastorianus* strains.

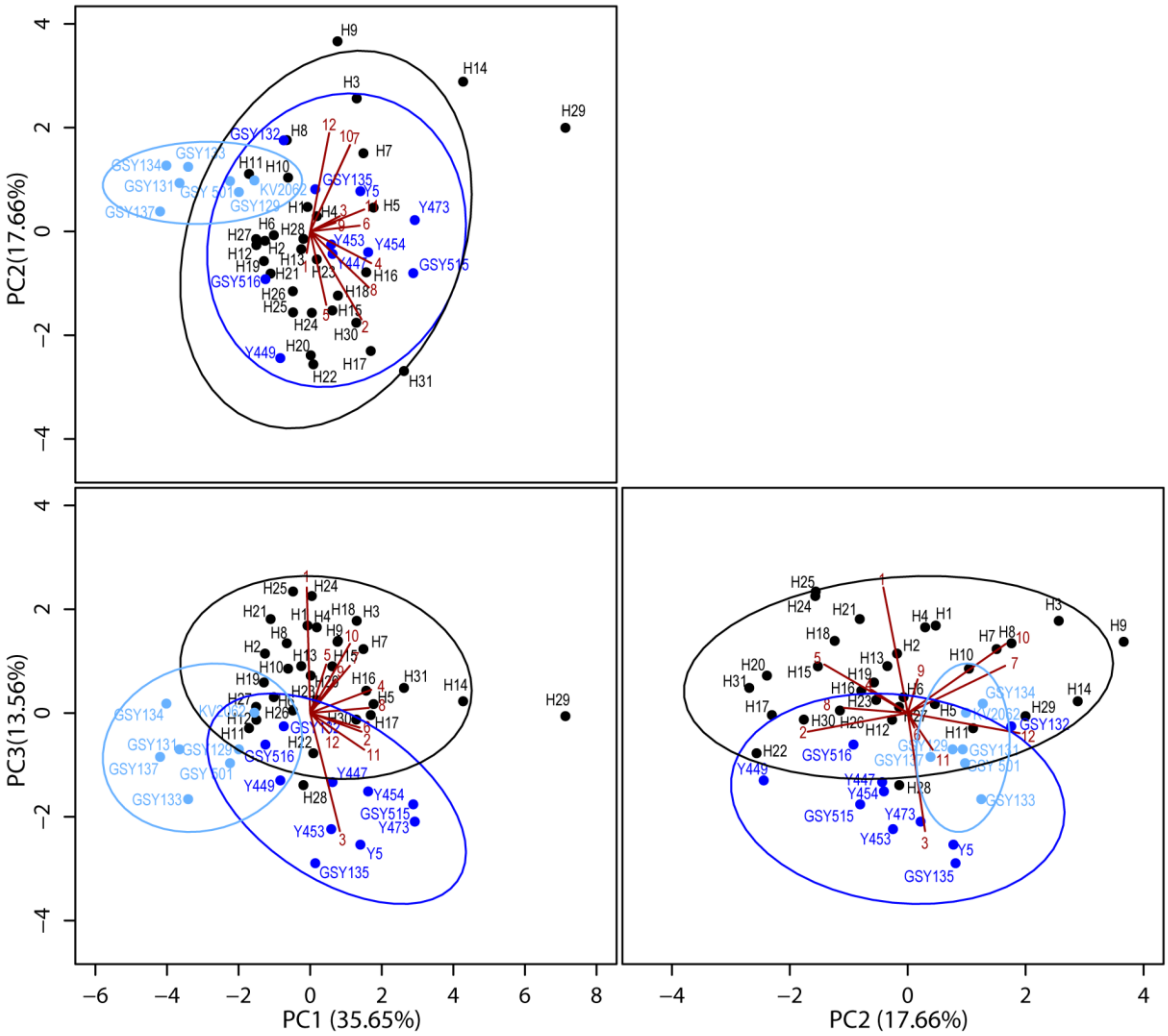

Figure 19: Three 2D-Principal Component Analyses (PCA), visualizing the aromatic diversity introduced by the newly developed interspecific hybrids (black spheres), seven reference Saaz type (light blue spheres) - and ten reference Frohberg type (dark blue spheres) S. pastorianus strains*. Ninety percent confidence ellipses are drawn in the corresponding colors. The component scores for the 12 aroma compounds are depicted as red lines with 1 = acetaldehyde; 2 = ethyl acetate, 3 = ethyl propionate, 4 = isobutyl acetate, 5 = ethyl butyrate, 6 = propanol, 7 = isobutanol, 8 = isoamyl acetate, 9 = butanol, 10 = isoamyl alcohol, 11 = phenylethyl acetate and 12 = phenyl ethanol. The three 2D-PCA plots represent 66.87% of the total variance of the dataset.*

The differences in aroma production were also confirmed by statistical analyses. Except for isobutanol and phenyl ethanol, the Kruskal-Wallis one-way analysis of variance test indicated stochastic dominance for the production of the ten remaining aroma compounds for at least one of the three groups (p-value < 0.05; Table S3), indicating a significant difference between the lowest and highest median production between the three groups for these compounds.

Furthermore, non-parametric post-hoc analysis revealed that Frohberg type reference *S. pastorianus* strains produced significantly higher concentrations of acetate esters such as ethyl acetate, isobutyl acetate, isoamyl acetate and phenyl-ethyl acetate compared to the Saaz type *S. pastorianus* reference strains, which is in line with previous findings (58, 178).

The interspecific hybrids in their turn produced significantly more acetaldehyde, ethyl butyrate and isoamyl alcohol and significant less ethyl propionate than the Frohberg type reference *S. pastorianus* strains (Dunn test with FDR corrected P-value < 0.01, Table S3). Interspecific hybrids were also significantly more aromatic than the Saaz type reference *S. pastorianus* strains, showing a higher production of esters like isoamyl acetate, isobutyl acetate, ethyl acetate and phenyl ethyl acetate. Additionally, they also produced significantly more fusel alcohols like isoamyl alcohol, propanol and butanol.

Lastly, the ability of the strains to produce 4VG, a compound associated with a phenolic or smoky flavour, was investigated (see paragraph 2.3 Materials and Methods for details). It was shown that all developed hybrids, as well as the *S. eubayanus* strains and 3/6 of the *S. cerevisiae* parents were able to produce this compound (Table S1). While production of this compound is generally undesired in lager beer, it was not detected sensorially in the small scale lager fermentations.

2.4.7 Pilot scale fermentation confirms the potential of new hybrids for commercial production of lager beer with a distinct aromatic profile.

To assess the industrial applicability of generated interspecific hybrids, the performance of four hybrids (H4, H5, H15 and H29) was tested in 50 L pilot scale fermentation and compared to the performance of their corresponding parental strains and two reference commercial *S. pastorianus* strains (GSY132 and GSY501). These four hybrids were selected based on their ploidy (two allodiploid hybrids and two allotriploid hybrids were included), their fermentation efficiency, and desirable aroma production in the lab scale fermentation experiments (Figure 17 and Figure 19).

Unfortunately, four out of the ten tested yeast strains (*S. eubayanus* Y567, hybrids H4 and H5 as well as the selected Frohberg type reference *S. pastorianus* yeast GSY132) yielded stuck fermentations and were unable to finish the fermentation within three weeks after pitching. These strains were therefore omitted from further analysis.

After fermentation and 10 days of cold storage at -0.5°C (lagering), the remaining six produced beers showed an ethanol content ranging from 4.93 to 5.19% vv^{-1}, with hybrid H29 producing the highest ethanol concentration (Table 3). The difference in ethanol production between strains was largely due to the inability of some strains to efficiently ferment the maltotriose present in the wort. Strains producing less than 5% ABV ethanol only fermented 50% (Y134, Y470, GSY501) or 60% (H15) of the maltotriose, whereas strains producing more than 5% ABV (Y397 and H29), fermented up to 70% of the maltotriose (Table 3).

Table 3: Overview of fermentation capacity (FC), aroma production and sugar consumption during pilot scale fermentation tests. *Fermentations conducted with strains GSY132, Y567, H4 and H5 were not finished within three weeks and where therefore omitted for further analysis. Values are the averages of two independent measurements. Concentrations in bold are those higher than the reported flavour threshold in lager beer (FTH; represented between brackets in second column)* (65, 104).

		GSY501	Y134	Y397	Y470	H15	H29
FC	fermentation time (days)	16	7	8	16	8	7
	ethanol production (%ABV)	4.93	4.94	5.13	4.92	4.98	5.19
Sugar consumption	glucose (%)	98.3	100.0	100.0	100.0	98.7	100.0
	maltose (%)	98.6	98.9	98.9	98.8	98.5	98.8
	maltotriose (%)	50.8	53.5	69.1	53.9	59.0	70.6
Aroma production	acetaldehyde (**10 mg.L^{1}**)	**11.01**	2.58	4.29	4.29	3.64	1.42
	ethyl acetate (**30 mg.L^{-1}**)	16.23	**34.22**	**36.34**	**30.83**	**38.59**	**30.21**
	ethyl propionate (**10 mg.L^{-1}**)	0.32	0.28	0.37	0.39	0.28	0.42
	isobutyl acetate (**1.6 mg.L^{-1}**)	0.08	0.22	0.33	0.20	0.18	0.24
	ethyl butyrate **(0.5 mg.L^{-1}**)	0.24	0.21	0.19	0.13	0.19	0.17
	propanol (**800 mg.l^{-1}**)	4.97	13.13	12.81	12.97	12.75	11.69
	isobutanol (**65 mg.L^{-1}**)	8.51	11.56	16.23	10.66	12.21	16.06
	isoamyl acetate (**1.2 mg.L^{-1}**)	**1.56**	**4.08**	**5.45**	**3.81**	**2.78**	**3.90**
	butanol (**50 mg.L^{-1}**)	N.D.	0.20	0.21	N.D.	0.19	0.21
	isoamyl alcohol (**70 mg.L^{-1}**)	33.29	54.66	**72.14**	49.44	51.84	**76.27**
	phenyl ethyl acetate (**3 mg.L^{-1}**)	2.44	**3.17**	**5.56**	2.69	**3.20**	**4.06**
	phenyl ethanol (**100 mg.L^{-1}**)	25.72	12.51	36.32	14.00	23.93	20.13

Sensory analysis by an independent tasting panel of the produced beer showed that the use of non-*S. pastorianus* strains increased the aromatic diversity and complexity of lager beers (see Table S4 for an overview of the sensory results). The reference Saaz-type *S. pastorianus*

strain GSY501 produced a lager beer with clear fruity and pineapple-like aromatic notes and was well appreciated by the panel (see Table S4). This fruity character of GSY501 is likely due to the relatively high concentration of isoamyl acetate.

S. cerevisiae parental strains produced overall complex and aromatic lager beers, characterized by a high production of isoamyl acetate and ethyl acetate (Table 3), with fruity notes in the aroma and/or taste. In addition to these aromas, strain Y470 introduced a slightly grainy and grassy aroma and taste into the beer, which was well appreciated by the panel. Strains Y134 and Y397 produced slightly sulphury notes, and an onion and sulphury off-flavour in the beers, respectively.

Strain H15 showed a similar fermentation capacity and a similar production of aroma compounds as compared to its *S. cerevisiae* parent Y134, but with a slightly lower isoamyl acetate production (2.78 compared to 4.08 $mg.L^{-1}$). The panel described this beer as having a slightly grainy aroma and grainy taste, characterized with slightly sulphury and metallic notes, resulting in an overall undesirable aroma.

Hybrid H29 displayed a faster fermentation and higher attenuation (i.e. higher final ethanol concentration) compared to its corresponding *S. cerevisiae* parental strain (Y397) and reference *S. pastorianus* yeast GSY501. Sensorial analysis of the beer produced with this strain revealed a complex fruity aroma profile. Indeed, chemical analysis revealed that strain H29 produced a high concentration of isoamyl acetate, ethyl acetate, isoamyl alcohol and phenyl-ethyl acetate, well above the respective flavour thresholds of 1.2, 30, 70 and 3 $mg.L^{-1}$. Therefore, despite the very slightly sulphury notes detected, the beer produced with this strain was highly rated by the tasting panel. Another interesting aspect to note is the low acetaldehyde production of this strain compared to its corresponding parental strain Y397and reference strain GSY501, which might increase the stability of the beer (144).

2.5 Discussion

Over the past decades, the beer industry is increasingly dominated by fewer firms (e.g. in 2012, 50 % of the beer sales and 70% of the revenues were accounted by only four breweries) (71). However, the past years have brought a remarkable increase in the demand for specialty beer, turning the global beer market into a niche market where product diversification has become pivotal. Despite the fact that the clean flavour and aroma of lager beer still remains

an important characteristic, new lager yeasts that can introduce aromatic diversity in Pilsner-type beers could be of considerable industrial importance and provide opportunities for breweries to expand their market share and diversify their product portfolio to fulfil the costumer's demands. Moreover, the generated interspecific hybrids can also be used to create a new niche beer market of aromatic, low alcohol, but still highly drinkable beers.

In this chapter, we had a closer look into the generation of 31 new interspecific hybrids through spore-to-spore mating of six carefully selected *S. cerevisiae* with two *S. eubayanus* strains. The overall yield of 1.5% obtained with the spore-to-spore mating technique is significantly higher than the yield obtained with mass mating approaches (e.g. hybridization frequency of 2.6×10^{-6} reported by Krogerus and coworkers (80)). Another advantage of the spore-to-spore approach is that no auxotrophic mutants of the parental strains are needed to be obtained prior to mating (80, 131). Moreover, because the experimental procedure only relies on natural mating and not on genetic modification, the generated interspecific hybrids are not considered to be GM organisms and can be used without restrictions by the beverage industry.

Interestingly, none of the 135 mating attempts between Y377 and Y565 yielded interspecific hybrids indicating that some strains within the *Saccharomyces* genus are less prone to mate when combined with a specific strain (62), and/or show more efficient prezygotic or postzygotic barriers due to strain-specific (and not species-specific) differences in spore germination timing (91, 97). Further research is needed to elucidate the underlying genetic base of this phenomenon.

Previous research showed that newly formed interspecific hybrid genomes are characterized by a high plasticity and that genome rearrangements such as whole or partial chromosome losses and introgressions are common and can be directed by the applied environmental stress conditions (41, 83, 111, 133). For example Dunn and coworkers (2013) showed that newly formed interspecific hybrids between *S. cerevisiae* and *S. uvarum* exhibited a characteristic genome rearrangement pattern when hybrids were grown under continuous ammonium limitations (41). The work of Piotrowski *et. al.* (2012) supports the hypothesis that the genomic fate of new interspecific hybrids depends on the stress they encounter in their immediate environment (133). Especially in lager production, where it is common practice to recycle yeast for several consecutive fermentations cycles (47, 135), it is paramount that these

strains remain genetically stable during the whole process. Therefore, genetic stabilization, based on vegetative growth in specific conditions mimicking typical lager beer fermentations, was a crucial step in the development of our strains (131).

The temperature tolerance profiles of the generated interspecific hybrids are in line with the results of Bellon and coworkers, who discovered a similar trend in interspecific hybrids between *S. cerevisiae* and the cold tolerant *S. mikatae* (15). Our results also line up with the recent findings of Hebly and coworkers, in which one generated interspecific hybrid between *S. cerevisiae* and *S. eubayanus* showed a similar temperature range as most of our generated interspecific hybrids (66). However, despite the industrial importance of cold tolerance, relatively little is known about the underlying molecular mechanisms. Therefore, the interspecific hybrids obtained in this study could be of use to further investigate the underlying genetic mechanisms of the observed cold tolerance.

In addition, our interspecific hybrids also showed interesting fermentation characteristics in lab-scale lager beer fermentation experiments. On average, the interspecific hybrids produced more ethanol compared to their corresponding best parental strain. These results are in line with the work of Peréz-Traves and coworkers and more recently by Krogerus *et al.*, in which interspecific hybridization between *S. cerevisiae* and cold tolerant yeast species such as *S. kudriavzevii* and *S. eubayanus* yielded variants able to outperform their parental strains in wine and beer fermentations at low temperatures (80, 131).

However, not all hybrids developed were able to outperform their respective parental strains in lager beer fermentations. This can be explained by a potential downside of the applied breeding strategy (spore-to-spore mating), where random haploid segregants of the parents are applied in the breeding experiments. Since industrial strains are often heterozygous, allelic segregation will cause a large diversity within the haploid segregants originating from the same parent, some of which might be inferior (156). Other breeding techniques, such as cell-to-cell mating, allow phenotyping of the haploid segregants prior to breeding, but, due to the homothallic nature the *S. eubayanus* parents, the cell-to-cell mating technique is not applicable.

Our results support the current hypothesis about the origin of lager yeasts, which states that the combination of the cold tolerance of *S. eubayanus* and the good fermentation capacity of

S. cerevisiae provided the interspecific hybrids a competitive advantage in ancient Bavarian lager beer production processes, typically conducted at very low temperatures (19, 42, 57, 66).

Moreover, some of the developed interspecific hybrids showed a higher fermentation efficiency than the best reference *S. pastorianus* strains that are currently used for commercial production (e.g. H15, H29 and H27) at 16°C, and also on pilot scale lager beer fermentation tests, conducted at 12°C (e.g. H29; which also showed the fastest fermentation and H15). Use of these interspecific hybrids in industrial lager beer production could potentially lead to a shortened fermentation time and consequently lead to higher profits.

Gibson and Liti (57) and, more recently, Krogerus and coworkers (80), hypothesized that the development of interspecific hybrids between *S. cerevisiae* and *S. eubayanus* could lead to aromatic diversity in lager beer production. This study provides the first proof for this hypothesis, showing that newly developed interspecific hybrids produced aromatic profiles that were significantly different from the aroma production of currently available lager yeasts, making them interesting new yeast strains for commercial lager beer production.

For example, strain H29, a hybrid of the ale-type *S. cerevisiae* Y397 and *S. eubayanus* Y567, outperformed its parents with respect to its fermentation capacity and fruity flavours production. This strain also performed extremely well at pilot-scale in a 50 L lager beer fermentation at 12°C, where it exhibited the fastest fermentation profile and reached the highest final ethanol titre. The resulting beer showed a complex fruity aroma and was highly appreciated by a trained, independent expert panel, further highlighting its industrial potential for the production of aromatic lager beer.

An interesting observation was the presence of smoky flavours in some, but not all hybrids and parental strains in pilot scale fermentation experiments. This sensorial attribute, typically associated with the presence of 4-VG, is an important factor in beer brewing, and is often negatively perceived in the beer industry (and therefore also called “phenolic off flavour” (POF)). Interestingly, our experiments indicate that while all our developed hybrids and most parental strains have the intrinsic potential to produce 4-VG (Table S1), it is only perceived in two pilot scale fermentations (H4 and *S. eubayanus* strains Y567). The strains for which no smoky flavour was detected, but do possess the potential to produce 4VG (hybrids H5, H15

and H29, as well as *S. cerevisiae* parent Y397), most probably produce this compound below the threshold level in Pilsner beer (300 ppb (167)), or it is masked by other flavour attributes, such as fruitiness.

In conclusion, we described the development of 31 novel interspecific yeast hybrids, resulting from large scale breeding experiments between six carefully selected *S. cerevisiae* strains and two feral *S. eubayanus* strains. The best newly generated hybrids showed growth at a broader range of temperatures, high fermentation capacity in lab scale lager beer fermentations, and desirable aromatic profiles that were significantly different from the profiles produced by the current applied lager yeasts. Importantly, these industrially interesting characteristics were also confirmed in pilot scale trials with hybrid H29 showing perhaps the most interesting profile, due to its combination of a fast fermentation, high attenuation (e.g. higher final ethanol concentration) and the production of a complex, desirable fruity aroma palate.

3

Development of a novel screening method for phenolic off-flavour production in yeast

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This Chapter is adjusted from:

Mertens, S., Steensels, J., Gallone, B., Souffriau, B., Malcorps, P. and Verstrepen, K.J. Rapid Screening Method for Phenolic Off-Flavour (POF) Production in Yeast. *J. Am. Soc. Brew. Chem.*, **75**: 318–323 (2017)

3.1 Abstract

Because of an increased interest in complex niche products and the ongoing quest for improved brewing efficiency and quality, the interest in novel brewing yeasts is increasing. However, many non-conventional yeasts and newly developed yeast hybrids often produce undesirable off-flavours, and in particular, are characterized by strong production of phenolic aroma's like 4-vinyl guaiacol (4VG). Here, we describe a novel high-throughput, low cost absorbance-based screening method that allows quick determination of the phenolic off-flavour production (POF) capacity of yeasts. We show that this method correlates well with the standard, more laborious GC-based methods, and that it can be used to assess POF production of many different strains in parallel with minimal hands-on labour. This new method therefore opens exciting new routes to select rare variants or mutations that combine desirable phenotypes without showing unwanted production of phenolic off-flavours like 4VG.

3.2 Introduction

During fermentation, yeast converts simple carbohydrates into its primary metabolites carbon dioxide, ethanol and glycerol, but also secondary metabolites, some of which are desirable, and others which are detrimental for the taste and aroma of the final fermentation product (156, 168, 170). The main aroma-active secondary metabolites produced by yeast are higher alcohols, aldehydes, sulphur-containing compounds, esters, phenols, carbonyl compounds and organic acids (160). Besides diacetyl and sulphur-containing off-flavours, phenolic off-flavours (POF), and most notably 4-vinyl guaiacol (4VG), are possibly amongst the prime unwanted compounds produced by yeast during beer production (except in a few styles like the German Hefeweizen and Belgian Wit beer, where some amount of 4VG is considered to be part of the style (167)) (165). Phenolic off-flavours like 4VG are characterized by a low flavour threshold (typically 0.2 to 0.4 mg/L) and are characterized by a medicinal or clove-like aroma that is highly undesirable in most beer styles.

4VG in beer is mainly formed by yeast via the decarboxylation of ferulic acid (167). Ferulic acid can be found in plant cell wall material, and is mostly associated with polysaccharides. During the brewing process, ferulic acid is both extracted and solubilized by hydrolases from the malt into the wort (33). In yeast, two genes (namely *PAD1* (728bp) and *FDC1* (1511bp); both

situated within the right subtelomeric region of chromosome IV for *S. cerevisiae*) are required for 4VG production. *PAD1* encodes for a flavin prenyltransferase (26.7 kDa), which catalyses the formation of a prenylated cofactor, required for the ferulic acid decarboxylase FDC1 (56.1 kDa)(29, 114).

In recent years, there is an emerging trend to explore new yeast variants in the brewing process. For example, many papers described how exploration of the natural yeast diversity can sometimes identify new, non-conventional species for beer brewing (14, 155, 156). Moreover, over the past decades, several strategies were developed to generate new beer yeast variants on a large scale (reviewed in (156)). Using these strategies, hundreds or even thousands of different yeast variants can be developed rapidly. Taken together these emerging trends have as a result that not the development, but rather the screening of these yeasts for specific brewing attributes, such as POF production, has become a major bottleneck.

Moreover, recent research demonstrated that except for two subfamilies of typical brewing yeasts, most yeasts do show strong production of 4VG. This excludes them from use in a mainstream brewery (54). Nevertheless, rare variants that do not produce 4VG are present, or can be obtained using breeding or mutagenesis. However, screening for these rare strains is cumbersome, and there is a strong need for a high-throughput method to assess the production of phenolic off-flavours.

Previous reports have proposed different techniques to determine the phenolic off-flavour of yeast. Traditionally, the different yeasts are grown in a ferulic acid containing medium or in brewing wort, where after production of 4VG is measured via sensorial analysis (149, 167), high pressure liquid chromatography (1, 10, 26, 46, 102, 167), gas chromatography (GC) (1, 54) or Nuclear Magnetic Resonance (NMR) spectroscopy (102). Unfortunately, all previously published methods are labour intensive and low-throughput techniques.

In this chapter, we report on the development and testing of a novel high-throughput and highly accurate method to determine the POF phenotype of hundreds of yeasts simultaneously. This method involves growth of yeasts in 96-well plates, a limited amount of hands-on-labour and requires only standard laboratory materials. The general strategy of this methodology is to measure absorbance of hydroxycinnamic acids before and after fermentation. Key to this strategy is that the absorbance of the main hydroxycinnamic acids

present in beer, namely ferulic acid and p-coumaric acid, does not overlap with the absorbance of their decarboxylated products (4VG and 4-vinyl phenol (4VP) respectively, for more details, see paragraph 1.3.5), nor with their further reduced products 4-ethylguaiacol and 4-ethylphenol (Table 4) (185). As a case study, we used this technique to evaluate the POF phenotype of 153 industrial *Saccharomyces* yeasts, and compared it to results obtained with a GC-based test.

3.3 Materials and Methods

3.3.1 Yeast strains.

In this study, 153 *Saccharomyces* yeasts were analysed for their POF production capacity. Strains were obtained from a yeast collection of the VIB Laboratory for Systems Biology (KU Leuven, Belgium) and White Labs (USA). More details concerning the geographical origin is listed by (54). In every test, a previously confirmed POF^+ and POF^- control were included (*Saccharomyces mikatae* NCYC2888 and *Saccharomyces pastorianus* type strain Weihenstephan 34/70 respectively). Furthermore, a set of three deletion mutants was generated, each with one or both POF related genes deleted (*PAD1*, *FDC1* and the double mutant).

3.3.2 Yeast transformation.

The wild type lab strain BY4741 was used to generate three deletion mutants (see above). A kanamycin (KANMX) selection marker cassette was amplified from the pym-n18 plasmid (74) using specially designed primers up- and down-stream of the gene we wanted to delete, each with an unique 50 bp sequence homologue to the DNA sequence, plus a 20 bp annealing sequence adjacent to the KANMX cassette on the plasmid. The polymerase chain reaction was conducted according the following regime; 30 repeats of 30 seconds at 95°C, 30 seconds at 57°C and 2 minutes at 72°C. In total, two primer pairs were designed to amplify the Kanamycin (KANMX) selection marker out of the pym-n18 plasmid (PAD1-FW: GATTTCAATCTACGGAGTCCAACGCATTGAGCAGCTTCAATTGAGTAGATCAGCTGAAGCTTCGTAC GC, PAD1-RV: ATCAACTCTATTAGTAGTTGAGTAACGTAATAAAATGCTCAGAAAAAGTGGTGCTTG-GGTGTTTTGAAGTGG, FDC1-FW: TGACATTATTACATCACCAATTCAAAAGAATTGTCAATTTATA-TATTTAACAGCTGAAGCTTCGTACGC and FDC1-RV: TATTTAAAATCTGATTATATGGTTTTTCT-TCCGTAGAAAGTCTATGGCAAGTGCTTGGGTGTTTTGAAGTGG). Because *PAD1* and *FDC1* are

situated next to each other on chromosome IV in *Saccharomyces cerevisiae*, the double deletion mutant was generated using an amplified maker with the PAD1-FW and FDC1-RV primers.

A standard lithium acetate-based yeast transformation protocol was used to generate the deletion mutants. Firstly, yeast was grown for one overnight in five mL YPD2% growth medium (yeast extract: 1 % w/v, peptone 2% w/v, glucose 2% w/v) at 30°C, 200 rpm, after which one mL of the pregrowth was transferred to 50 mL YPD2% growth medium and incubated for an extra four hours (30°C, 200 rpm). Next, the yeast cell culture was centrifuged (3 minutes at 3000 rpm) and cells were resuspended into 200 µL 0.1 M lithium acetate solution. After 10 minutes incubation at room temperature, 50 µL of the cell culture were mixed with 40 µL PCR product, 300 µL PLI (142 M Polyethylene glycol, 0.12 M lithium acetate, 0.01 M Tris (pH7.5) and 0.001M EDTA) and 5 µL salmon sperm DNA (1mg.mL^{-1}) and incubated for 25 minutes at 42°C. Cells were centrifuged (3 minutes at 3000 rpm) and resuspended in fresh YPD2%, after which cells were recuperated for one overnight on YPD2% agar plates (yeast extract: 1 % w/v, peptone 2% w/v, glucose 2% w/v, agar 1.5% w/v) at 30°C. Selection of true deletion mutants was done via replica plating onto selective agar plates (YPD2% + geneticin (0.2 µg.mL^{-1}), followed by a two day incubation at 30°C. Selected deletion mutants were confirmed via sanger sequencing.

3.3.3 Absorbance-based POF measurement.

Strains were inoculated from -80°C on standard YPD2% agar plates (yeast extract: 1 % w/v, peptone 2% w/v, glucose 2% w/v, agar 1.5% w/v). Subsequently, individual colonies were inoculated into 150 µL of standard growth medium (YPD2%) supplemented with 100 mg/L ferulic acid (Sigma Aldrich, Belgium). The 96-well plates were sealed with an aluminium sticker and incubated for 5 overnights at 30°C, shaking (900 rpm). After incubation, plates were centrifuged (3 min, 3000 rpm) and 100 µL supernatant was transferred into a new 96-well plate. Next, absorbance was measured at 325 nm wavelength, using a 96-well OD measurement device (tecan infinite pro m200, Advanced Bioscience). During all experiments, positive and negative controls were included (see above), as well as blank measurements (non-inoculated YPD2% + ferulic acid medium). Each strain was tested in biological and technical replicates. Strains were regarded as POF$^+$ if the measured amount of ferulic acid was below the 90% confidence interval of the blank.

3.3.4 Comparison gas chromatography-based and absorbance-based POF production screening tools.

Similar to the absorbance-based POF measurement, strains were inoculated from -80°C stock on to standard YPD2% medium. Subsequently, individual colonies were inoculated in a GC vial, filled with 5 mL standard growth medium (YPD2%; yeast extract: 1 % w/v, peptone 2% w/v, glucose 2% w/v) supplemented with 100 $mg.L^{-1}$ ferulic acid. Vials were capped (but not completely closed) and wrapped with paraffin and statically incubated for 5 overnights at 30°C. Afterwards, 200 µL was sampled into a 96-well plate and further analysed according to the absorbance-based POF measurement protocol. The concentration of 4VG was measured using HS-GC-FID as described earlier (54). Strains were regarded as POF^+ if the measured amount of post-fermentation ferulic acid was below the 90% confidence interval of the blank (YPD2% + 100 $mg.L^{-1}$ ferulic acid without any yeast inoculated) or if the produced concentration 4VG was above the 90% confidence interval of the blank.

3.3.5 Data analysis and representation.

All statistical tests were conducted in Graphpad Prism (Graphpad Software Inc., San Diego), whereas the figures were generated in R (163), using the ggplot2 package.

3.4 Results

3.4.1 Conversion of ferulic acid into 4VG by yeast can be accurately measured using absorbance.

Previous research has shown that hydroxycinnamic acids absorb light around 300 nm to 330 nm wavelength and that this does not overlap with the absorbance of their decarboxylated products (see Table 4) (12). Hence, we hypothesized that the conversion of these aroma precursors into aroma molecules could be followed by measuring a decline in absorbance at these wavelengths. While the results summarized in Table 4 confirms that the proposed assay can indeed be used to detect conversion between any hydroxycinnamic acid and their hydroxystyrene, we will focus in this paper on measuring the conversion of ferulic acid to 4VG, which is generally recognized as the most influential POF in beer (167).

Table 4: Overview maximal absorbance peaks for ferulic acid and p-coumaric acid, together with their representative vinyl- and ethyl- derivatives

Compound	**λ_{MAX} (nm)**
Hydroxycinnamic acids	
ferulic acid	321, with shoulder at 275 (124)
p-coumaric acid	285, with second lower peak at 305 (12)
Hydroxystyrenes	
4-vinyl guaiacol	223 and 256.5 (185)
4-vinyl phenol	200 and 256 (185)
Ethyl derivates	
4-ethyl guaiacol	230 and 280 (25)
4-ethyl phenol	220 and 278 (25, 161)

To find the optimal wavelength at which ferulic acid can be quantified, we determined the absorbance of different concentrations of ferulic acid (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2, and 0 $mg.L^{-1}$) dissolved in water or YPD2% growth medium over a wide span of wavelengths (295 to 400 nm, intervals of 1 nm). As shown in Figure 20, absorbance measurements at 325 nm are highly correlated with increasing ferulic acid concentrations. Indeed, a good linear correlation was obtained between the measured absorbance at 325 nm wavelength and the added concentrations ferulic acid in water ($R^2 = 0.9964$, $p < 0.0001$) and more importantly, in YPD2% ($R^2 = 0.9952$, $p < 0.0001$).

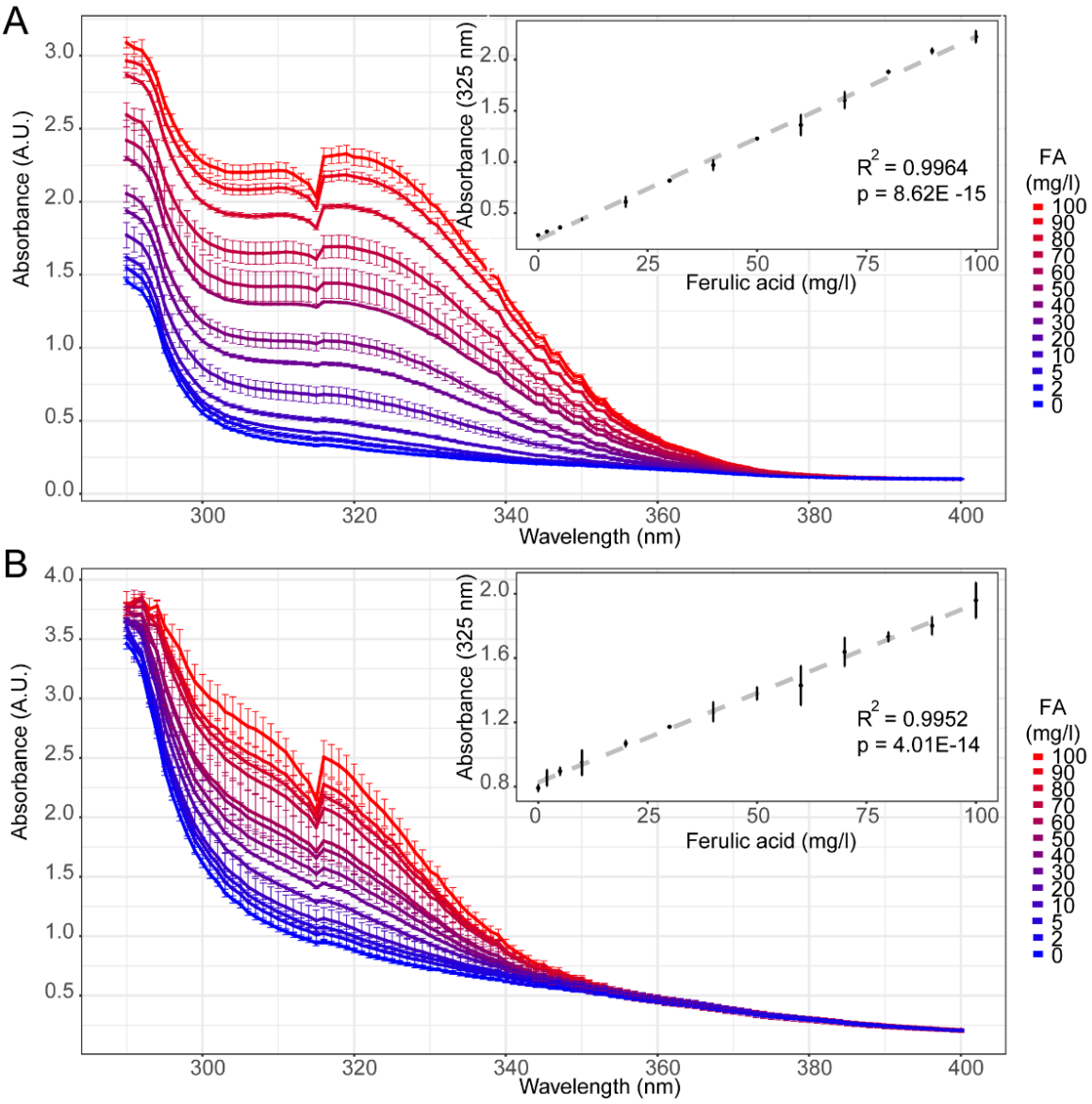

Figure 20: Absorbance profile of different concentrations of ferulic acid, measured in water (A) and YPD2% growth medium (B). *Different ferulic acid concentrations were prepared, ranging from 0 mg.L^{-1} to 100 mg.L^{-1} in water (A) and YPD2% growth medium (B). Their corresponding absorbance profiles, measured at a wide range of wavelengths (from 290 nm to 400 nm, with steps of 1 nm) are depicted, as well as a scatterplot showing the measured absorbance of the different concentrations at 325 nm wavelength. Error bars represent standard deviations. FA = ferulic acid. A.U. = arbitrary units.*

3.4.2 Post-fermentation ferulic acid measurements anti-correlate with 4VG production.

Next, we determined whether the concentration of ferulic acid that remains after fermentation with yeast correlates with the amount of the phenolic off-flavour 4VG produced. To this end, 20 strains were grown in YPD2% supplemented with 100 mg.L^{-1} ferulic acid. After a 5-day fermentation at 30°C, leftover ferulic acid was determined using the developed absorbance-based assay and compared to the produced amount of 4VG, measured with HS-

GC-FID. As depicted in Figure 21, a highly significant anti-correlation was observed between both measurements (R^2 = 0.87, p- value < 0.0001), suggesting that the post-fermentation concentration of ferulic acid is a good measure for 4VG production.

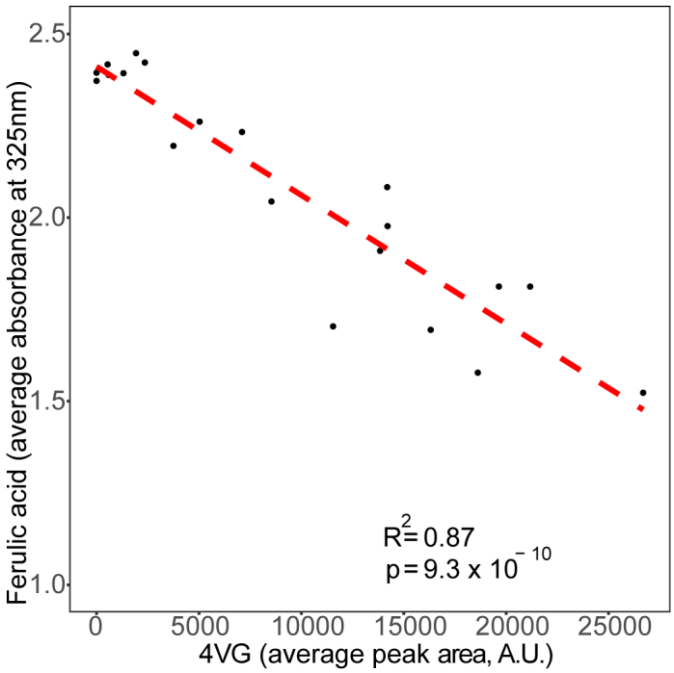

Figure 21: 4VG production and left-over ferulic acid is anti-correlated. *Represented is the correlation between the amount of 4VG and the leftover amount of ferulic acid measured after fermentation of 20 yeast strains. The red dotted line represents a linear trend line. The corresponding Pearson correlation coefficient and p-value are depicted on the figure. A.U. = arbitrary units.*

3.4.3 Confirmation of the test by POF+ and gene inactivated POF- yeasts.

Next, we investigated the ability our absorbance-based method to differentiate between POF^+ and gene inactivated POF^- yeasts. To this end, three yeast deletion mutants were generated in the POF^+ lab strain 'S288c', one in which *PAD1* was deleted, one in which *FDC1* was deleted, and one in which both genes were deleted. It has been described that deleting one or both of these genes results in a loss of the POF phenotype (54, 103). The strains were inoculated in YPD2% supplemented with 100 $mg.L^{-1}$ ferulic acid and incubated for 5 days at 30°C. As expected, a significant absorbance difference was observed between the POF^+ lab strain and the absorbance of the deletion mutants at 325 nm ($p < 0.01$; Figure 22). Moreover, the average absorbance of the deletion mutants was not significantly lower than the blank values (growth medium without yeast inoculated), indicating that no ferulic acid is consumed by these mutant yeasts, whereas this is the case for the wild yeast variant. The obtained results prove that the proposed technique indeed can be used to differentiate between POF^+ and POF^- yeasts.

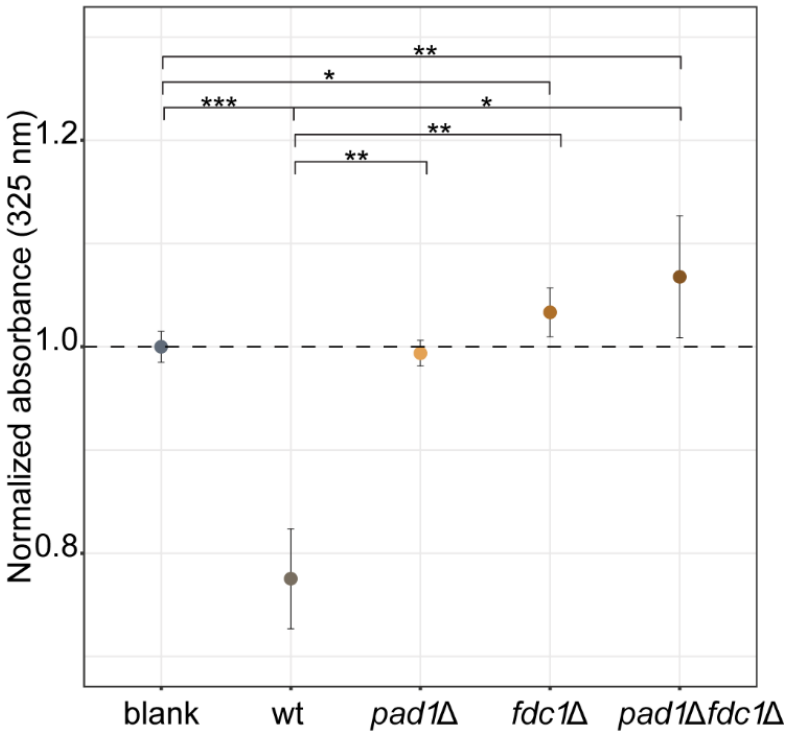

Figure 22: Absorbance measurement deletion mutants at 325 nm. *Depicted on the figure is the average relative absorbance (relative to the average absorbance of the blanks) of the wild type (POF$^+$) strain and of generated deletion mutants. Error bars represent standard deviations. Asterisks on the figure represent obtained p-values from unpaired t-test analysis (* p-value < 0.01; ** p-value < 0.001; *** p-value < 0.0001).*

3.4.4 Rapid evaluation of the POF phenotype in a large set of 153 industrial strains.

Next, we investigated the throughput and accuracy of the developed method by screening a set of 153 industrial and wild *S. cerevisiae* yeasts for their POF phenotype. The selected strains are a representative set of the present-day diversity of industrial yeasts, and include 100 beer isolates, as well as 46 isolates from other industries (wine (19), sake (7), spirits (11), bakery (4), biofuel (5)) and 7 natural isolates (54). Overall, less than two hours of hands-on-labour and 70 mL of medium was involved in this test, indicating the ease and low cost of the developed method.

In Figure 24, an overview of the results obtained, as well as a comparison with a previously conducted GC-based POF screening is presented (54). We obtained a good correlation between the output of both individual screenings (R^2 = 0.73, p-value < 0.0001, Figure 23). When taking the 90% confidence interval of the blank measurements as cut-off, both tests attribute the same phenotype for 147 out of the 153 (= 96%) tested yeast strains. The remaining yeast strains all scored POF$^+$ based on the results obtained with the absorbance

method, while they were classified as POF$^-$ with the GC-based method. Interestingly, these strains bear no loss of function mutations in *PAD1* and *FDC1*, and are expected to be POF$^+$, which might indicate that the absorbance-based method likely provides higher resolution compared to the standard GC-based method.

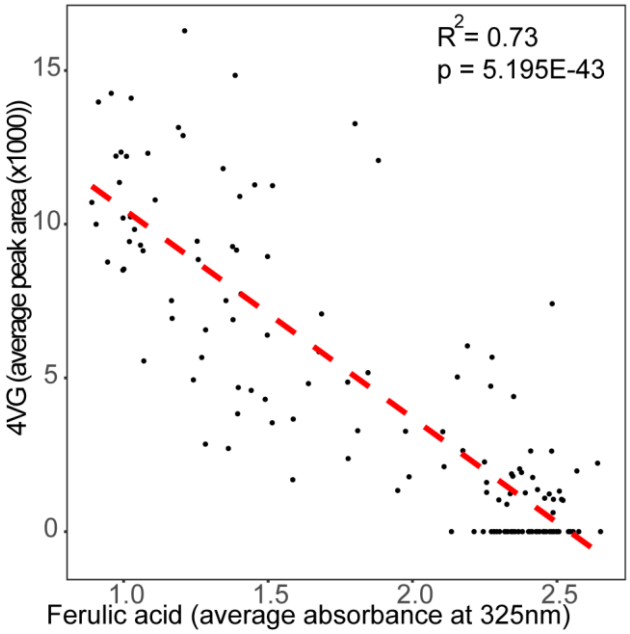

***Figure 23**: GC-based method is anti-correlated with Absorbance-based POF measurement* *represented is a correlation plot, comparing the produced amount 4VG for 150 tested yeast strains (GC-based method; (43)) and the post-fermentation amount of ferulic acid, measured with the absorbance based technique. The red dotted line represents the obtained linear trend line. The corresponding Pearson correlation coefficient and p-value are depicted on the figure. A.U. = arbitrary units.*

Both screening assays are in concordance with the predicted genotype in 129 cases (= 84%). The results obtained with the absorbance based screening are in agreement with the predicted phenotype based on the sequence of *PAD1* and *FDC1* for 135 tested strains (= 88%), whereas this is only true for 129 strains when taking the GC screening results into account. Interestingly, 18 strains are scored POF$^-$ by both tests, whereas they do not carry any loss-of-function mutations in either *PAD1* or *FDC1.* These strains are likely still able to convert ferulic acid into 4VG, but at levels well below the detection limit of both methods. Another possible explanation could be that these strains bear mutations in the promoter of *PAD1* or *FDC1* or in other genes, impairing their ability to perform the decarboxylation of ferulic acid into 4VG (1). Overall, the proposed absorbance based POF screening is a good and rapid measure to determine the POF phenotype of large yeast collections or yeast segregants.

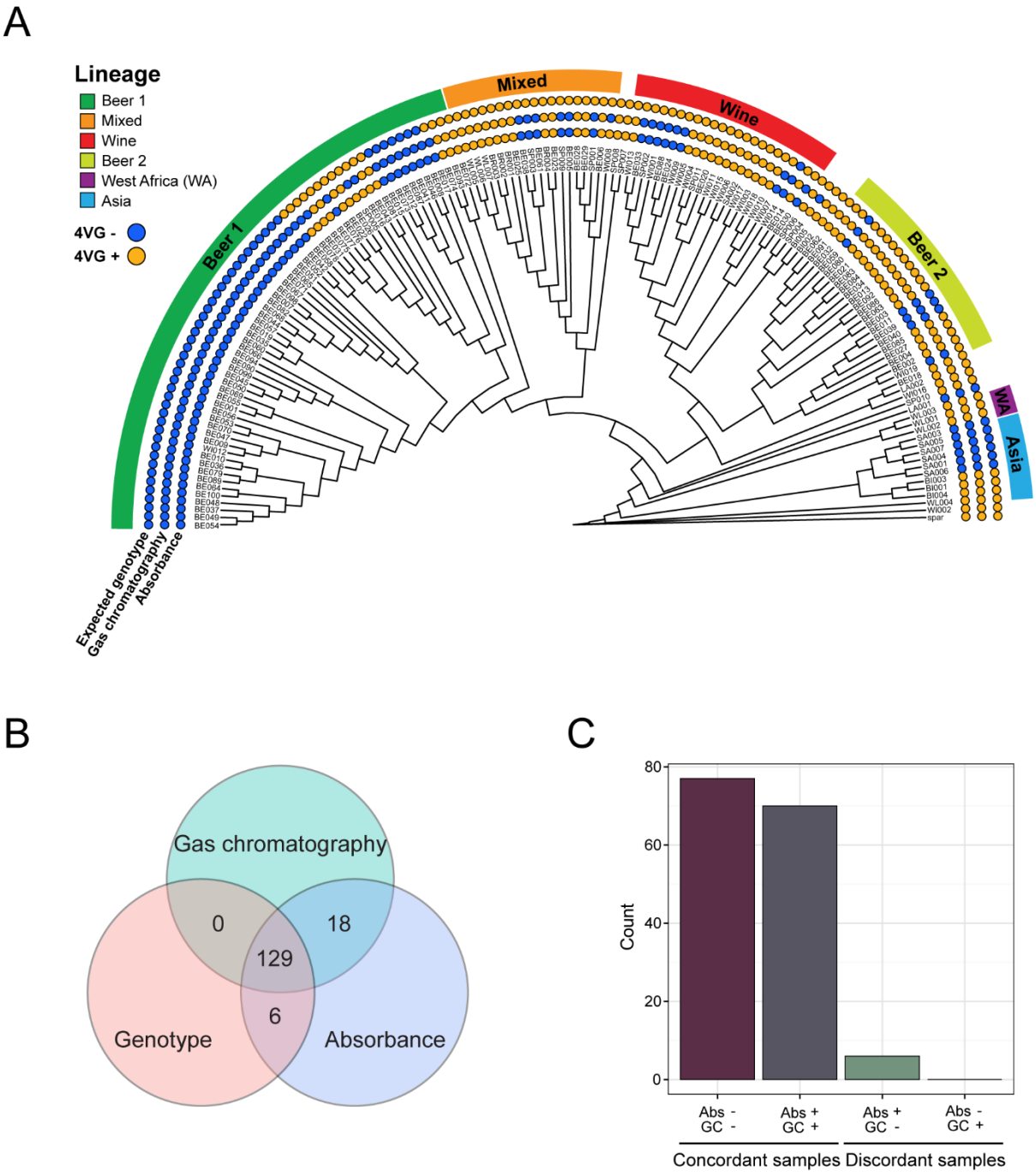

Figure 24: Comparison of the absorbance-based POF screening method with the GC-based method and their expected phenotype based on their genotype. *(A) visual representation of the obtained results of both the GC- and absorbance-based screenings of the strain set. In addition, a prediction of the phenotype based on the sequence of* PAD1 *and* FDC1 *is given ('Expected Genotype'). The clustering of the strains is based on whole-genome sequence data of the strains. When a non-sense mutation in all copies of either* PAD1 *or* FDC1 *is present, the genotype is scored POF⁻. The coloured outer bands depict to which genetic lineage these strains belong. For a thorough description of the strain set and details on the genetic clustering, see* (54). *(B) Venn diagram, showing the similarity between the two screening assays and the genotype-based predicted POF phenotype. (C) Details on the direct comparison of the GC- and absorbance-based screening methods (Abs = absorbance).*

3.5 Discussion

The proposed new screening method described allows determining the POF phenotype of many yeasts in parallel, with only a limited amount of labour, consumables or expensive machines. As a case study, a set of 153 yeasts strains was screened for their POF phenotype within less than two hours of hands-on-labour. It is also likely that the new method based on measuring the post-fermentation amount of ferulic acid is more precise than the currently used GC-based method, as ferulic acid is less volatile than 4VG, which limits the risk of false negatives. In addition to the detection of 4VG-producing *Saccharomyces* yeasts, the absorbance of ferulic acid, p-coumaric acid and their corresponding vinyl- and ethyl-derivatives do not overlap, indicating that the proposed test can also be used to, for example, screen the 4-ethyl guaiacol and 4-ethyl phenol production capacity of *Brettanomyces* and other non-*Saccharomyces* yeasts. Therefore, this method can provide a rapid and inexpensive tool for high-throughput screening of a large number of yeasts, for example large mutant libraries or collections of non-conventional yeasts, either in an academic or industrial setting.

4

CRISPR-cas9 based gene editing of novel generated interspecific yeast hybrids to reduce off-flavour production in lager beer

This chapter is adapted from:

Mertens, S., Gallone, B., Steensels, J., Herrera, B., Cortebeek, J., Nolmans, R., Saels, V., Vyas, V.K. and Verstrepen, K.J. (2018). Reducing phenolic off-flavours through CRISPR-based gene editing of the *FDC1* gene in *Saccharomyces cerevisiae* x *Saccharomyces eubayanus* hybrid lager beer yeasts. PloS one, accepted manuscript.

4.1 Abstract

Today's beer market is challenged by a decreasing consumption of traditional beer styles and an increase in the consumption of specialty beers. In particular, lager-type beers (pilsner), characterized by their refreshing and unique aroma and taste, yet very uniform, struggle with their sales. The development of novel variants of the common lager yeast, the interspecific hybrid *Saccharomyces pastorianus*, has been proposed as a possible solution to address the need of product diversification in lager beers. Previous efforts to generate new lager yeasts through hybridization of the ancestral parental species (*S. cerevisiae* and *S. eubayanus*) yielded strains with an aromatic profile distinct from the natural biodiversity. Unfortunately, next to the desired properties, these novel yeasts also inherited unwanted characteristics, most notably phenolic off-flavour (POF) production, which hampers their direct application in the industrial production processes. Here, we describe a CRISPR-based gene editing strategy that allows the systematic and meticulous introduction of a natural occurring mutation in the *FDC1* gene of genetically complex industrial *S. cerevisiae* strains, *S. eubayanus* yeasts and interspecific hybrids. The resulting cisgenic POF^- variants show great potential for industrial application and diversifying the current lager beer portfolio.

4.2 Introduction

Although interspecific hybridization (i.e. hybridization between two different species) is believed to be rare in nature, next generation sequencing recently revealed the presence of several interspecific hybrid yeasts within the *Saccharomyces* yeast clade (23, 60, 139). While some hybrids have occasionally been isolated from natural habitats, most isolates were obtained from man-made industrial environments (24, 40). The best-known and most-studied example of such interspecific hybrid is *Saccharomyces pastorianus*, the yeast species used for lager beer production. This hybrid yeast originated from a cross between the common ale beer and baker's yeast *Saccharomyces cerevisiae* and the cold-tolerant species *Saccharomyces eubayanus* (57, 89). The resulting *S. pastorianus* hybrid combines the ability of *S. cerevisiae* to efficiently ferment sugars in beer wort with the cold-tolerance of *S. eubayanus*, making it the ideal yeast to perform lager beer fermentations, which are typically performed at lower temperatures (42, 57, 136). Besides *S. pastorianus,* other hybrid types are associated with industrial environments, such as *S. cerevisiae* and *S. kudriavzevii* hybrids (isolated from ale

beer and wine fermentations), and *S. cerevisiae* X *S. uvarum* hybrids, which are sometimes isolated from wine fermentations (28, 60, 61).

The discovery of an increasing number of interspecific hybrids in industrial fermentation processes inspired researchers to mimic the hybridization in the lab, often with the aim of generating new variants that would expand the existing spectrum of industrial yeasts (56). Hybridization between different strains of *S. cerevisiae* strains has proven an effective method to generate new variants with interesting industrial properties (152, 155, 156), and the ability to include non-*cerevisiae* strains in the breeding schemes further broadens the gene pool and thus the phenotypic diversity of the resulting hybrids. Over the past years, multiple reports describe hybridization between *S. cerevisiae* strains and *S. uvarum* (117, 147, 148), *S. eubayanus* (66, 80, 105, 98, 117), *S. kudriavzevii* (4, 15) or *S. arboricola* (117). The newly generated interspecific hybrids often show hybrid vigor in their fermentative capacity, broadened temperature tolerance and/or diversified metabolite and aroma profiles. Interspecific hybrids therefore open new routes to address changes in the global fermented beverage market, including an increasing demand for low alcohol, high-flavour and unique products (56, 157).

While newly formed interspecific hybrids combine interesting characteristics of their respective parental species, they can also inherit undesired phenotypes that impede their direct implementation in industrial production processes. More specifically, the non-*cerevisiae* strains are less adapted to industrial fermentations and as a result they often perform poorly in the specific stress conditions imposed by industrial environments. Moreover, the feral yeasts often produce certain undesirable aroma compounds. Arguably the most important drawback of the feral species is the production of phenolic off-flavours (POF), most notably 4-vinyl guaiacol (4VG) (80, 105, 117). In beer, 4VG is usually an undesirable yeast metabolite that imposes a very distinct spicy, clove-like flavour. It is produced by yeast through the bioconversion of ferulic acid, present in the endosperm of the malt and barley, to its decarboxylated derivative, 4VG (113, 167). The genetic underpinnings of this phenotype are well-described, and involve the action of Fdc1p and Pad1p (113, 167). The first decarboxylates ferulic acid, while the latter provides a prenylated Flavin-mononucleotide (FMN) cofactor of Fdc1p, required for its function.

Several successful strategies have been described to obtain artificial interspecific yeast hybrids that are POF$^-$. First, after the hybrid has been formed, an additional step consisting of a backcross to the POF$^-$ *S. cerevisiae* parent effectively removes the phenotype. However, newly formed interspecific hybrids are usually sterile due to the postzygotic barriers between members of the *Saccharomyces* clade, which limit interspecific hybrids to a vegetative lifestyle (97, 157). Interestingly though, recent publications have found a way to circumvent this hybrid sterility. A first approach is the generation of allotetraploid interspecific hybrids via rare mating of a diploid *S. cerevisiae* strain with a diploid non-*cerevisiae* yeast cell (2, 81, 147). The resulting allotetraploid interspecific hybrids can form viable allodiploid spores which could be used for backcrossing with spores of its POF$^-$ parental strain or with another yeast. This approach allowed Krogerus and coworkers to generate a POF$^-$ interspecific yeast hybrid, combining genetic material of three parental strains (81). Nevertheless, this approach also has some insurmountable drawbacks. It relies on a rather complex breeding scheme, on the generation of auxotrophic mutants of the candidate parental yeasts, and on two consecutive rounds of breeding that require extensive screening of the segregants.

A second approach involves direct modification of the non-*cerevisiae* parent to eliminate the POF phenotype using mutagenesis (36). Segregants of the *S. eubayanus* parental strain are subjected to UV mutagenesis and subsequently screened to identify POF$^-$ mutants that can be directly applied in a breeding scheme. However, this strategy also has important limitations, most notably the large screens required to identify positive mutants and the risk of off-target mutations with potential undesired phenotypic effects. Despite the availability of a high-throughput screening method for POF production (106), identification of a POF$^-$ mutant with no or a very limited number of mutations in other genes would require a screening setup with an even higher throughput.

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated protein (Cas9)-based genome editing technology offers a new tool to modify phenotypes of industrial Saccharomyces yeasts (99, 145). For example, a CRISPR-based genome editing strategy successfully reduced urea production in wine yeasts (172) and introduced the hops monoterpenes biosynthesis pathway in an ale brewing yeast, yielding yeast cells that produce certain hop aromas (34). Moreover, a recent publication describes an optimized CRISPR strategy to alter the genome of the industrial S. pastorianus yeasts CBS1483

and W34/70, which allows to efficiently alter specific phenotypes such as ester production, by knocking out the responsible genes (174).

While CRISPR-based technologies demonstrate a broad spectrum of potential applications, the legislation surrounding the use of cisgenic gene-edited organisms differs widely between different countries across the planet. A recent EU ruling states that these organisms should follow the same guidelines as other genetically-modified organisms. By contrast, other countries, including Brazil, USA, Japan and Argentina, have installed specific guidelines for the use of CRISPR-based gene editing that in some cases allow such modified organisms to be used without following the GM legislation, which greatly increases their industrial potential (72, 121, 179).

In this chapter, we report a CRISPR-based gene editing strategy to develop cisgenic POF- variants of genetically complex industrial yeasts and interspecific hybrids by introducing a naturally occurring loss-of-function mutation in the FDC1 gene. We applied and optimized this strategy for S. cerevisiae strains with varying ploidy, a non-cerevisiae species (S. eubayanus), and newly developed interspecific lager yeasts. We show that our strategy allows the introduction of homozygous mutations, resulting in cisgenic mutants that lost the ability to produce POF without undesirable side effects.

4.3 Material and methods

4.3.1 Yeast used in this chapter

Yeast parental strains for the generation of interspecific hybrids were selected from a collection of 301 industrial and wild *Saccharomyces* yeasts, previously characterized by Steensels and coworkers (155). *S. cerevisiae* strains SA003 and BE011 were selected based on their POF pheno- and genotype, as well as for their ability to form viable spores (54, 155). Additionally, *S. eubayanus* strains WL2022 and WL024 were selected based on their temperature tolerance and sporulation efficiency and viability (105). *S. cerevisiae* strains BE002, BE014, BE020 and BE074 were selected as candidate strains to test the proposed CRISPR based genome editing strategy in industrially relevant *S. cerevisiae* strains.

Table 5 Overview yeast strains used in this chapter

Strain	Species	Industry	Origin
S288C (n)	*S. cervisiae*	Lab	(6)
S288C(n)_A	*Gene edited S. cervisiae*	Lab	This study
S288C (2n)	*S. cervisiae*	Lab	(6)
S288C(2n)_A	*Gene edited S. cervisiae*	Lab	This study
SP003	S. cerevisiae	Saké	Japan
BE011	*S. cerevisiae*	Beer	Belgium
BE002	*S. cerevisiae*	Beer	Bulgaria
BE002_A	Gene edited *S. cerevisiae*	Lab	This study
BE014	*S. cerevisiae*	Beer	Belgium
BE014_A	Gene edited *S. cerevisiae*	Lab	This study
BE014_B	Gene edited *S. cerevisiae*	Lab	This study
BE014_C	Gene edited *S. cerevisiae*	Lab	This study
BE020	*S. cerevisiae*	Beer	Belgium
BE020_A	Gene edited *S. cerevisiae*	Lab	This study
BE020_B	Gene edited *S. cerevisiae*	Lab	This study
BE020_C	Gene edited *S. cerevisiae*	Lab	This study
BE074	*S. cerevisiae*	Beer	Germany
BE074_A	Gene edited *S. cerevisiae*	Lab	This study
WL022 (NPCC1286) [a]	*S. eubayanus*	Wild	Argentina
WL022_A	Gene edited *S. eubayanus*	Lab	This study
WL024 (NPCC1292) [a]	*S. eubayanus*	Wild	Argentina
WL024_A	Gene edited *S. eubayanus*	Lab	This study
W34/70	*S. pastorianus*	Lager	Germany
NCYC2888 [b]	*S. mikatae*	Wild	Japan
H1	Interspecific hybrid (BE011 X WL022)	Lab	This study
H1_A	Gene edited H1	Lab	This study
H1_B	Gene edited H1	Lab	This study
H1_C	Gene edited H1	Lab	This study
H1_D	Gene edited H1	Lab	This study
H2	Interspecific hybrid (SP003 X WL022)	Lab	This study
H2_A	Gene edited H2	Lab	This study
H2_B	Gene edited H2	Lab	This study
H2_C	Gene edited H2	Lab	This study
H2_D	Gene edited H2	Lab	This study

[a]NPCC: North Patagonian Culture Collection, Neuquén, Argentina

[b]NCYC: National Collection of Yeast Cultures, Quadram Institute Bioscience, Norwich, UK.

4.3.2 Interspecific hybrid generation through spore to spore mating and hybrid-state confirmation

Interspecific yeast hybrids were generated by the previously described spore to spore mating technique (105). First, parental strains were subjected to a random spore isolation protocol (152). Next, a micromanipulator (MSM-singer instruments) was used to pair two spores on a YPD2% agar plate (2%[wt vol^{-1}] Bacto peptone,1%[wt vol^{-1}] yeast extract, 1.5%[wt vol^{-1}] and 2%[wt vol^{-1}] glucose), one from each parental strain. After six to eight hours of incubation at room temperature, the formation of a zygote ('Shmoo') was investigated. Possible hybrids were further purified by restreaking (3x) the strains on synthetic 12°P wort medium (Light spray malt extract: 13%[wt vol^{-1}]; Agar: 1.5%[wt vol^{-1}]). The hybrid nature of the possible hybrids was confirmed via a species specific multiplex PCR, as described in previous research (105). PCR-confirmed interspecific hybrids were prolonged stored at -80°C to ensure strain purity. Afterwards, generated hybrids were genetically stabilized according to the previously reported stabilization protocol (105).

4.3.3 CRISPR Cas9-based gene editing

Description plasmid:

A *S. cerevisiae* compatible version of the *Candida albicans* solo vector CRISPR system, previously described by Vyas and coworkers (176), was used as a platform for the CRISPR based gene editing (177). The *S. cerevisiae* and *C. albicans* optimized CAS9 endonuclease (CaCas9, where the use of the 'CUG' leucine codon is avoided, which is predominantly translated as serine by CTG clade species like *C. albicans* and *S. cerevisiae*), as well as the single guide (sg)RNA were introduced into the yeast shuttle vector pR5416, which provides a CEN/ARS sequence for plasmid maintenance in yeast (150). Both the CaCAS9 and the sgRNA are preceded by a constitutive promoter (respectively TEF1p and SNR52p). Species specific sgRNA sequences (*S. cerevisiae* 5-GGCAAGTACTTACAAACGTA-3'; *S. eubayanus* 5'-GGCAAGTATTTGCAAACGTA-3') were cloned into the vector as described previously (176).

Repair templates:

Double stranded (ds) DNA oligo's were created as repair template for the homology directed repair of the induced DSB. Each of the repair templates are 100 nucleotides long, and are

centered around the induced DSB in the yeast DNA and contained desired mutations in its sequence. The 100 nucleotides long repair templates were generated via a PCR fill-in of two 60bp long primers with an overlap of 20 nucleotides at their three prime end (primer sequence, see Table S14)

Transformation:

A standard lithium acetate-based yeast transformation protocol was used to transform both the CRISPR plasmid, as well as the repair template into the target strains (106). Firstly, yeast was grown for one overnight in 5 mL YPD2% growth medium at 30°C, 200 rpm, after which 1 mL of the pregrowth was transferred to 50 mL YPD2% growth medium and incubated for an extra 4 hours (30°C, 200 rpm). Next, the yeast cell culture was centrifuged (3 minutes at 3000 rpm) and cells were resuspended into 200 µL 0.1 M lithium acetate solution. After 10 minutes incubation at room temperature, 50 µL of the cell culture was mixed with 500 ng plasmid, in which the corresponding sgRNA was cloned and 5 to 25 µg (adjusted protocol) repair template DNA, 300 µL PLI (142 M Polyethylene glycol, 0.12 M lithium acetate, 0.01 M Tris (pH7.5) and 0.001M EDTA) and 5 µL salmon sperm DNA (1mg.mL^{-1}) and incubated for 30 minutes at 42°C. Cells were centrifuged (3 minutes at 3000 rpm) and resuspended in fresh YPD2%, after which cells were recuperated for one overnight on YPD2% agar plates at 30°C. Selection of plasmid-containing cells was done via replica plating onto selective agar plates (YPD2% + clonat (0.2 µg.mL^{-1}), followed by a 2 day incubation at 30°C. Growing colonies were subjected to a second round of selection on YPD2% + clonat agar plates or immediately plated on YPD2% agar plates (adjusted protocol). After selection, strains were grown for three consecutive rounds onto YPD2% agar plates in order to induce plasmid loss, prior to long term storage at -80°C.

Introduction of the correct mutation was determined via Sanger sequencing with species-specific primers (see Table S14 for an overview of used primers).

4.3.4 Absorbance-based POF measurement.

The ability of the yeasts to produce POF was tested via the absorbance based detection method, described previously (106). Yeasts were inoculated in 150 µL liquid YPD2% growth medium, supplemented with 100 mg.L^{-1} ferulic acid in a 96 well plate. In each plate, a POF$^-$ (W34/70) and a POF$^+$ (*S. mikatae* NCYC2888) control were included. 96-well plates were sealed with an aluminum sticker and incubated for 5 days at 30°C, 200 rpm. After centrifugation (3

min, 3000 rpm), 100 µL of the supernatant was transferred to a new 96 well plate and remaining concentration of ferulic acid was measured at a wavelength of 325 nm (Tecan Infinite® 200 PRO, Switzerland). Yeasts were regarded as POF^- if the absorbance at 325 nm was above the lower limit of the 90% confidence interval of the POF^- control (W34/70).

4.3.5 Laboratory-scale lager fermentations.

Lab scale fermentations were performed according a previously described protocol (105). First, yeast was propagated by inoculation into 5 mL YPD2% medium at room temperature and 300 rpm. After 16 h of incubation, 1 mL of the culture was transferred to 50 mL 4% yeast extract-peptone-maltose (YPM;2%[wt vol^{-1}] Bacto peptone,1%[wt vol^{-1}] yeast extract, and 4%[wt vol^{-1}] maltose) in a 250 mL Erlenmeyer flask and incubated at 20°C and 200 rpm for 3 overnights.

Cell concentration of the pregrowths was measured (BioRad, TC20® automated cell counter, USA), and the calculated amount of cells was used to inoculate 150 mL of an 16°P wort (17% [wt.vol^{-1}] Light spray malt extract, Brewferm, Belgium, supplemented with 0.005 mg.L^{-1} zn^{2+}, autoclaved for 10 minutes at 110°C) to a starting concentration of 1.5 X 10^7 cells.mL^{-1}).

The 250 mL bottles were equipped with a water lock and stirring bar after which they were incubated at 14°C, agitated at 150 rpm. Weight loss was measured on a daily basis to track fermentation kinetics. Fermentations were stopped when the daily weight loss was equal or less than 0.05 g. Next, the fermentations were cooled on ice to prevent evaporation of the volatile compounds, and samples for chromatographic analysis (HS-GC-FID, Shimadzu corporation), ethanol (Alcolyzer beer ME, Anton Paar GmbH), sulfite and glycerol (Gallery Plus Beermaster, Thermo Scientific) measurements were taken. The leftover fermented medium was used for sensory analysis.

4.3.6 Data analysis and representation

All data analyses and visualization were performed in R (163). Statistical analyses were conducted within the multicomp package (version 1.4-8 (70)). Figures were generated using the ggplot2 package (version 2.2.1 (183)).

4.4 Results

Our aim was to develop and test a CRISPR-based genome editing strategy aimed at modifying the POF phenotype of industrial yeasts, including polyploid and aneuploid yeasts as well as

interspecific hybrids. More specifically, we aimed at introducing a loss-of-function SNP mutation in the *FDC1* gene that occurs naturally in many domesticated industrial ale beer yeasts (54).

4.5 Identification of a CRISPR target region for modification of the POF phenotype

The first step in CRISPR-based gene editing is finding an appropriate target region and designing the necessary sgRNA sequence (99). This region needs to meet some basic requirements. First, the region should harbor a loss-of-function mutation present in the natural biodiversity of *Saccharomyces* yeasts. Introducing such mutation in the same species or a closely related one is regarded as cisgenic modification, which will favor its industrial applicability. Second, the region should be highly conserved between different strains and even species, ensuring that the strategy works in several yeast strains, species and hybrids. Third, the region should contain a neighboring PAM DNA sequence, essential for the correct identification by the Cas9 endonuclease of its target site. Fourth, the region should also be unique in the genome to avoid off-target activity.

Analysis of the genome sequences of industrial POF^- *S. cerevisiae* yeasts, as described by Gallone and coworkers (54), indicates that disruption of either *PAD1* or *FDC1*, inhibits POF production (106, 113). The majority (73.80%) of POF^- strains from the 'Beer 1' lineage (54) share a C to T mutation at position 460 of the coding sequence of *FDC1*, that replaces a glutamine residue (CAA) by a stop codon (UAA) (Figure 25). This premature stop codon likely disrupts the protein's function, as both the dimerization domain as well as the catalytic pocket of the protein are not formed anymore (Figure 26) (18). More importantly, for the other species within the *Saccharomyces* species complex with a known *FDC1* sequence, the same C to T mutation at position 460 of the *FDC1* coding sequence introduces a similar stop codon (UAA) (Figure 25).

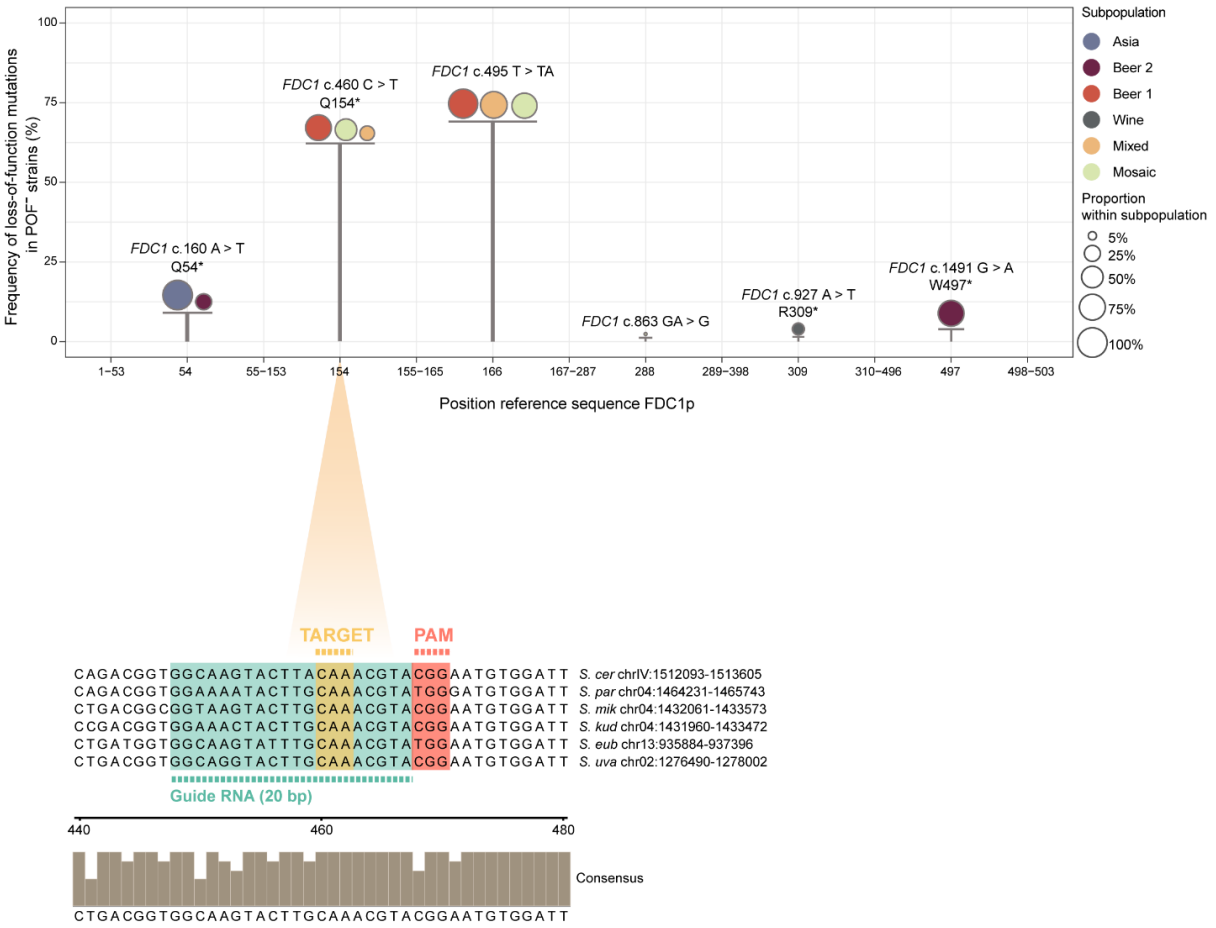

Figure 25: ***Natural loss-of-function mutations identified in the FDC1 gene and selection of target mutation*** (A) Occurrence of natural loss-of-function mutations in the *FDC1* gene across a collection of 76 POF⁻ *S. cerevisiae* strains (54, 106). Bars indicate the presence and the position of the mutation in Fdc1p based on *S. cerevisiae* s288C reference sequence. Height of the bars indicate the frequency of the mutation across the full collection of POF⁻ *S. cerevisiae* strains considered. Distribution of the mutation is further dissected across *S. cerevisiae* subpopulations (circles - colors) and its proportion within each subpopulation is summarized (circle - size) (exact frequencies are reported in S1 Table). Type and position of the mutation in the coding sequence are annotated on top of each bar. Only POF⁻ *S. cerevisiae* strains that harbor homozygous loss-of-function mutations were included in the analysis. (B) Natural *FDC1* loss-of-function point mutation selected for the CRISPR-Cas9 gene editing procedure (yellow cone). The alignment represents a zoom-in of the targeted region in the coding sequence of *FDC1* across six *Saccharomyces* species (-20nt, +20nt from the targeted point mutation). Colored boxes highlight specific areas of the targeted region: the targeted glutamine codon (CAA) that will be replaced by a stop codon (TAA) (yellow box), the PAM sequence (red box) and the guide RNA (green box). The bar-chart represents the alignment of consensus annotation for each position in the targeted region as calculated by Jalview (181).

Moreover, there is a high degree of conservation in the DNA sequence surrounding this mutation in all 156 previously sequenced *S. cerevisiae* strains (54). Indeed, the sequence of the proposed sgRNA sequence is 100% identical for 155 out of the 156 sequenced *S. cerevisiae*

strains. In addition, a PAM sequence (NGG) could be found in the close proximity of this mutation (9 nucleotides downstream of the mutation), which is crucial for the proper guidance of the CaCAS9 endonuclease to its target (Figure 25) (176). Importantly, the same PAM sequence, situated 9 nucleotides downstream of the target mutation site, can be found in the *FDC1* sequence of all other sequenced members of the *Saccharomyces* species complex, allowing the design of a possible sg RNA sequence for these *Saccharomyces* species (Figure 25).

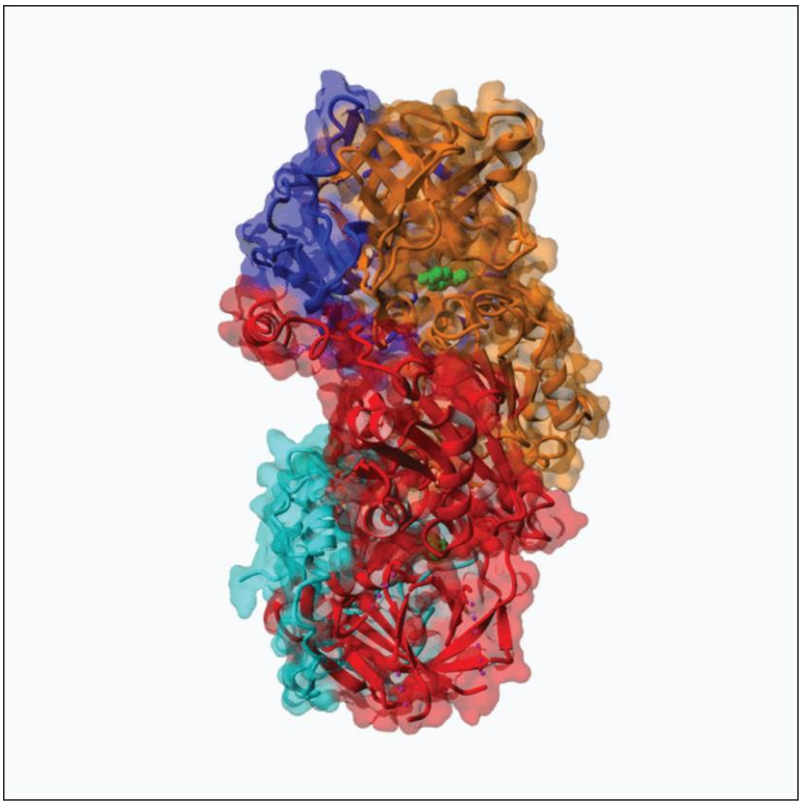

Figure 26: 3D crystal structure of FDC1. *FDC1 acts as a dimer (blue and red form one monomer, cyan and orange, the second one).Blue and cyan parts of the molecules represent the N-terminal part of the protein that it still formed before the stop-gain mutation p.154, whilst the red and orange parts disappears when Q154 is replaced by a stop codon* (18)*. Two 4VG molecules, which are bound to the catalytic pockets of both monomers, are colored in green.*

Lastly, possible off-target reactions of the CRISPR system were assessed by blasting the newly designed species-specific 20 nucleotide guide sequences plus possible PAM sequence (NGG) against the genome of the 156 previously sequenced *S. cerevisiae* yeasts (54), as well as against the de-novo assembly of the *S. eubayanus* genome (8).This analysis shows that the proposed sgRNA sequences are species- and target unique as no other sequences with more than 85% similarity were detected. Moreover, mismatches or gaps of off-target sequences

with the highest similarity happen in the first 13 bp immediately upstream of the PAM sequence, which has been shown to be sufficient to achieve a 100% off-target free gene editing in *Saccharomyces* yeasts (73).

Together, this indicates that the proposed region (Figure 25) is an appropriate candidate for effective cisgenic CRISPR based engineering of the POF phenotype in pure and hybrid *Saccharomyces* species.

4.5.1 Evaluation of CRISPR efficiency in euploid *S. cerevisiae* and non-*cerevisiae* yeasts

Gene editing of industrial *Saccharomyces* yeasts is complicated by two main factors. First, many industrial yeasts are poly- and/or aneuploid, and therefore can contain multiple alleles of the target genes, which all need to be modified by the CRISPR system. Analysis of the genomes of industrial ale yeasts shows that these strains have an average ploidy level of 3.52, with some yeasts showing a ploidy level above 4. As POF production is a dominant trait, all alleles of *FDC1* or *PAD1* need to be deactivated to affect the phenotype. Furthermore, CRISPR protocols are generally optimized for lab strains of *S. cerevisiae*, and their efficiency for editing non-*cerevisiae* or mixed genomes can be low (73, 99, 177).

To assess the efficiency of CRISPR in polyploid genomes, we introduced the desired nonsense mutation in *FDC1* in different POF^+ *S. cerevisiae* strains with different ploidy levels. Besides a lab strain (haploid and diploid S288c), POF^+ industrial beer yeasts BE014 and BE020 (diploid) and BE002 and BE074 (triploid) were subjected to the CRISPR transformation. These strains contain two (BE014, BE020) or three (BE002, BE074) functional copies of the *FDC1* gene, and at least one functional *PAD1* gene (54).

Overall, a decrease in the efficiency of CRISPR gene editing was observed with increasing ploidy levels and genome complexity. The haploid and diploid strains showed a high success rate similar to that of the laboratory strains. Introduction of the mutations was observed in 100% (8/8) and 87.5% (7/8) of the investigated colonies for the haploid and diploid variant, respectively. For both diploid beer yeasts, the introduced mutation was homozygous in 100% (10/10 and 5/5 respectively) of the tested colonies. Efficiency in BE074 and BE002 was lower, with homozygous mutations observed in 50%(1/2) and 10% (1/10) of the tested colonies, respectively.

To evaluate the efficiency of the strategy in non-*cerevisiae* species, we introduced the same C to T mutation in the *FDC1* gene of two POF$^+$ diploid *S. eubayanus* yeasts (WL022 and WL024) (FIG 1). This gene editing was highly successful, with an efficiency of 100% (3/3 and 1/1 respectively) for both strains, yielding POF$^-$ variants that may be suitable for industrial application (36, 56).

Evaluation of the newly formed mutants revealed that the introduction of the homozygous nonsense mutation effectively abolish the ability to produce the unwanted POF aroma (Figure 27).

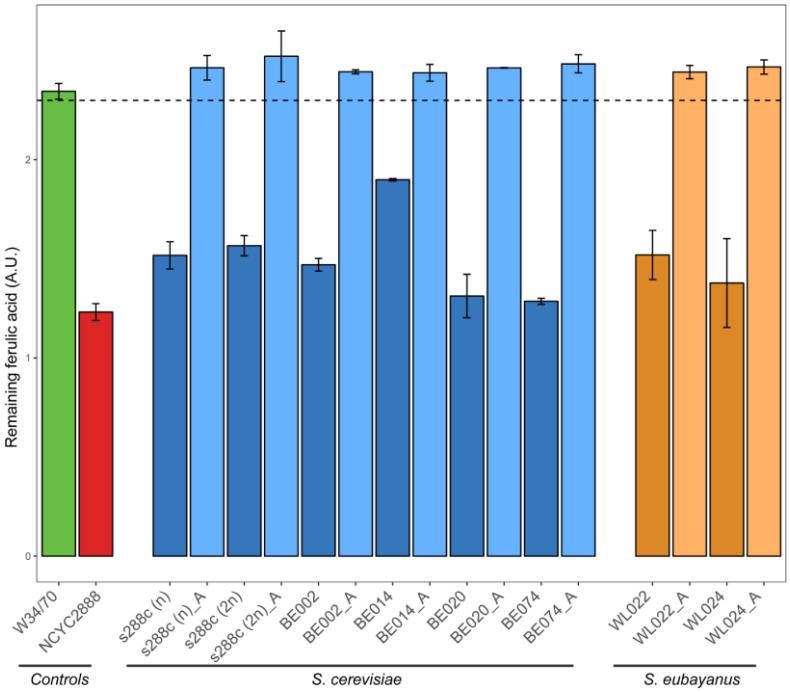

Figure 27: CRISPR mutants lose their ability to convert ferulic acid to 4VG. *Yeast cultures were incubated with an excess of ferulic acid, the precursor for the POF aroma. A decrease in ferulic acid indicates POF aroma formation. Remaining ferulic acid was measured for two control strains (POF- control (green); POF+ control (red)), six different S. cerevisiae strains (dark blue) and a gene edited variant of each (light blue), as well as two S. eubayanus yeasts (orange) and a gene edited variant of each (light orange). Error bars represent the standard deviation of two biological replicates. The dotted line represents the applied cut-off value (lower border of the 90% confidence interval of the POF-control).*

4.5.2 Gene editing of interspecific hybrid yeasts can induce loss of chromosomal fragments

Next, the CRISPR based gene editing was evaluated in interspecific hybrids. Therefore, we first generated POF hybrids between *S. cerevisiae* and *S. eubayanus*, after which we tried to

remove their POF production by introducing a nonsense mutation in their *FDC1* genes using the developed CRISPR strategy.

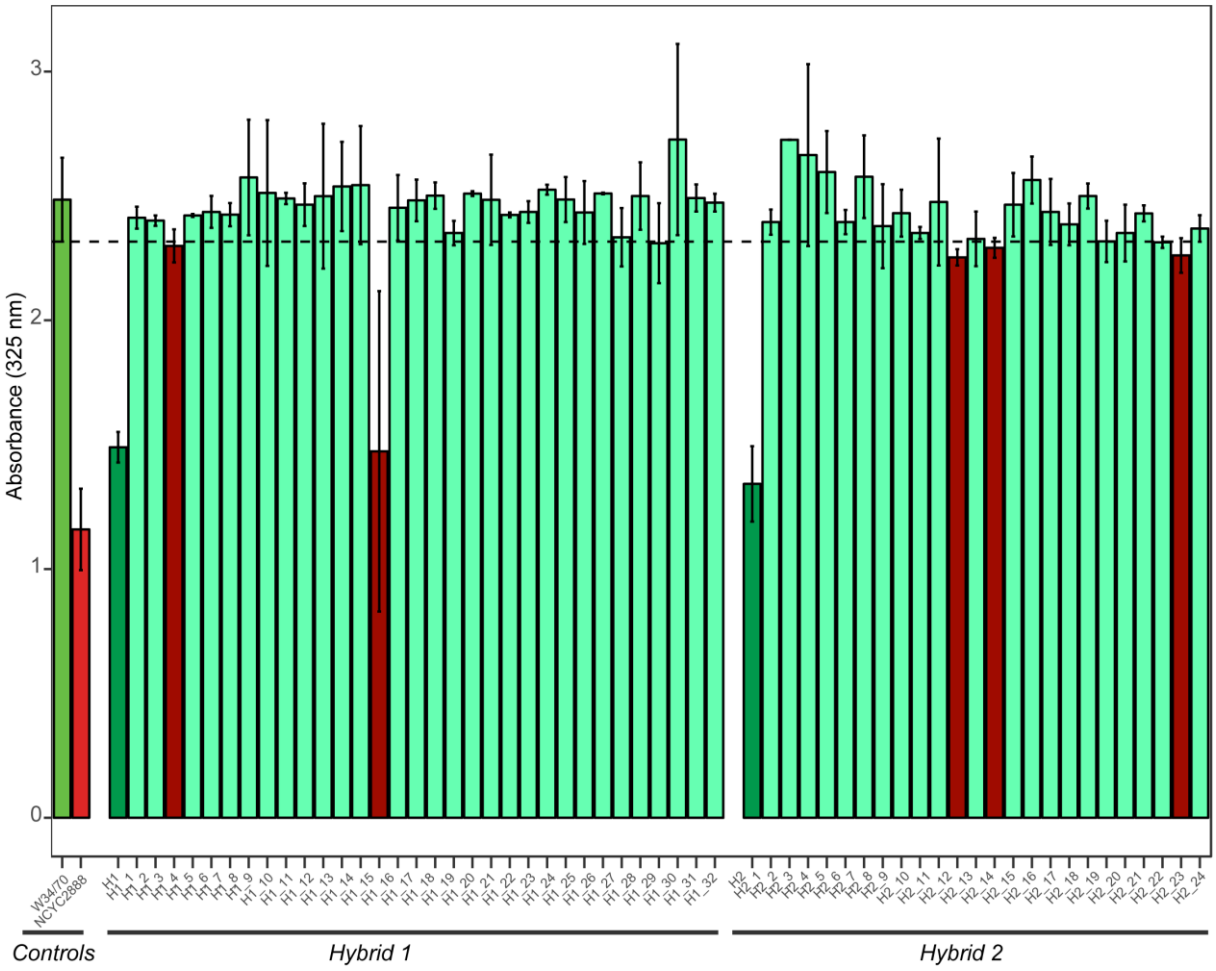

Figure 28: POF phenotype CRISPR variants with standard protocol*. Remaining ferulic acid was measured for two control strains (POF- control (green); POF+ control (red)), Interspecific hybrid one and two (dark green) with their respective POF- (light green) and POF+ (dark red) CRISPR variants, obtained via the standard CRISPR protocol. Error bars represent the standard deviation of two biological replicates. Dotted line represents the used cut off value (lower border of the 90% confidence interval of the POF-control).*

Initially, the standard CRISPR-based gene editing strategy (177) was used to introduce the selected SNP mutation in the *S. eubayanus* derived *FDC1* allele of the novel generated interspecific hybrids H1 and H2. The *S. cerevisiae* parent of H1 (BE011) is POF$^-$ and its *FDC1* carries a homozygous nonsense mutation in *FDC1* p.W497* (54)s. The *S. cerevisiae* parent of H2 (SP003) is also POF$^-$ and its *FDC1* is heterozygous for the nonsense mutation p.Q154* and homozygous for the insertion T>TA at position p.166 (54). As these mutations differ from the one targeted in the developed CRISPR-based gene editing strategy, it allows to determine the

species-specificity of the designed strategy towards the alleles derived from their *S. eubayanus* and *S. cerevisiae* parents.

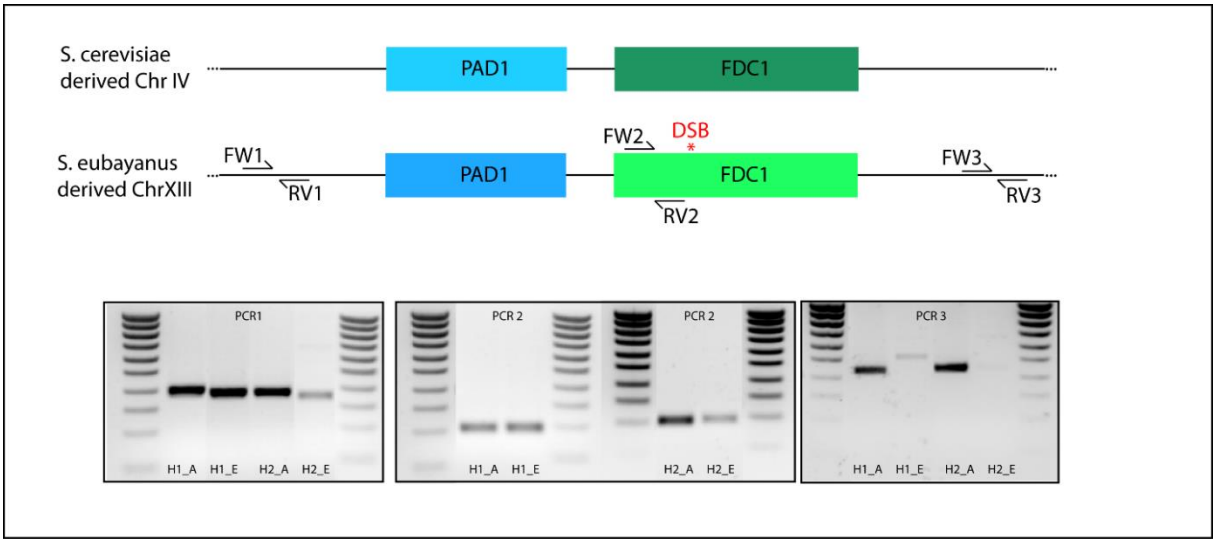

Figure 29: Standard CRISPR protocol causes genetic instability in complex hybrid genomes*. Three primer pairs were designed, each targeting a different part of the S. eubayanus derived Chr13 in the generated interspecific hybrids H1 and H2. Primer pair one and two amplify a region 4kb and 8b upstream of the introduced DSB respectively. Primer pair three amplifies a region 1kb downstream of the introduced DSB on the S. eubayanus derived chromosome of our hybrids. Primer pair three does not yield any PCR product for gene edited variants generated with the standard CRISPR protocol (H1_E and H2_E) but does for gene edited variants generated with the adjusted CRISPR protocol (H1_A and H2_A), proposed within this chapter.*

For the first interspecific hybrid, H1, 30 POF^- variants were obtained out of 32 tested (94%). Similarily, H2 yielded 21 POF^- variants out of 24 (87.5%) (Figure 28). However, control PCR reaction (primer pair SS_FWSE and SS_RVSE (Table S14)), which amplifies 490 bp surrounding the DSB induced in the *S. eubayanus* derived *FDC1* gene, did not yield an amplification product. Interestingly, further genetic characterization of this region revealed that the CRISPR editing induced the loss of the region downstream of the targeted region in the *S. eubayanus* Chr13 (Figure 29).

Genomes of artificial interspecific hybrids are notoriously unstable, and introduction of a double-stranded break likely caused partial loss of the respective chromosome (42, 95, 133). Whereas this genomic rearrangement yields POF^- variants, it is undesirable as it might have major unwanted pleiotropic consequences for other phenotypes. Therefore, the strategy was further optimized to eliminate this detrimental effect in interspecific hybrids. Increasing the concentration of repair template from 5 to 25 µg during the transformation stage and

shortening the selection step for the presence of the CRISPR plasmid from two rounds to only one round of selection, allowed us to overcome this interspecific hybrid genome instability. This strategy yielded POF$^-$ variants (Figure 30), with a 12.9 (H1, 4/31) and 6.84% (H2; 8/117) efficiency. None of these variants showed partial chromosome loss. Moreover, none of these variants acquired the targeted mutation in the *S. cerevisiae FDC1* allele (confirmed via Sanger sequencing), showing the specificity of the designed CRISPR-based genome editing strategy.

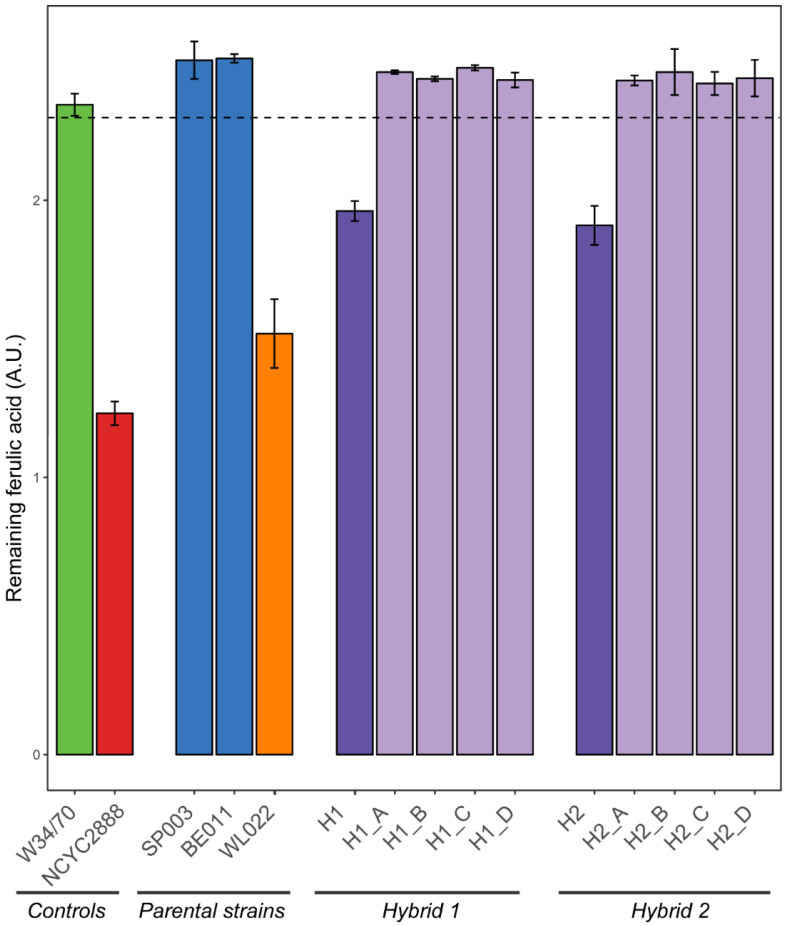

Figure 30: CRISPR mutants lose their ability to convert ferulic acid to 4VG. *Remaining ferulic acid was measured for two control strains (POF$^-$ control (green); POF$^+$ control (red)), S. cerevisiae and S. eubayanus parental strains (dark blue and orange, respectively), Interspecific hybrid H1 and H2 (dark purple) with four of their respective CRISPR variants (light purple). Error bars represent the standard deviation of two biological replicates. The dotted line represents the applied cut off value (lower border of the 90% confidence interval of the POF$^-$ control).*

4.5.3 Gene edited variants do not show phenotypic side-effects.

To determine whether the gene-editing procedure introduced any unwanted side-effects to the fermentation performance of the yeasts, we evaluated their performance in lab-scale beer fermentations and compared the profiles to those of their respective wild types (WT).

Overall, profiles of the various gene-edited variants were similar to those of their wild-type ancestral strains, with no significant differences in CO_2 production throughout the fermentation ((Figure 31A, Table S6), with the exception of strain WL022, where the mutant showed a faster fermentation at T2 (Anova-test; P-value <0.001) and T3 (P-value <0.01)). Furthermore, no significant differences were measured in ethanol production at the final stage of the fermentation (Table S7 – S12).

At the end of the fermentation, 17 different flavour-active metabolites were evaluated. Apart from the targeted phenotype, namely the production of 4VG (see further), no differences between gene edited variants and the WT were observed (Anova and post-hoc Tukey test; P-value > 0.05; Table S7 – S12), except for *S. eubayanus* WL024, where a significant difference was measured for one aroma compound, isoamyl alcohol production (P-value = 0.004; average isoamyl alcohol production of WL024 is 227.34 $mg.L^{-1}$ and 253.85 $mg.L^{-1}$ for WL024_A). Principal component analysis (PCA), based on the production of the remaining 16 metabolites, shows a clear clustering of the different gene-edited variants with their respective wild-type ancestors, further confirming that apart from the production of POF aroma, the overall flavour profile of the strains remained unchanged compared to that of the parental wild-type strains (Figure 31B).

As expected, all mutants showed a dramatic decrease in 4VG production ((Figure 31C, P-values ranging from <0.001 to 0.033; Table S7 – S12), with the 4VG concentrations remaining below or around the reported flavour threshold of 0.3 $mg.L^{-1}$ (167).

Subsequent sensorial analysis by a trained panel supported the above-mentioned measurements. Whereas BE014 and BE020 clearly produced clove like off-flavours, the fermentation products obtained with the respective gene-edited variants were described as ‘very fruity’ and ‘neutral’. Similarly, mutants of *S.eubayanus* strains WL022 and WL024 were described as ‘slightly fruity’ to ‘fruity’. H1’s aroma was defined as being ‘slightly fruity’ with ‘phenolic’ notes. All four gene edited variants of H1 were scored similar by the panel as being

‘very fruity’, highlighting the masking effect of 4VG on fruitiness. A similar trend was observed for H2. Interestingly, the aromatic contribution of all generated gene-edited variants was still remarkably different than the aroma produced by the reference lager yeast W34/70, revealing the potential of these hybrids to broaden the aromatic diversity of lager beers, without introducing unwanted clove like phenolic off-flavours.

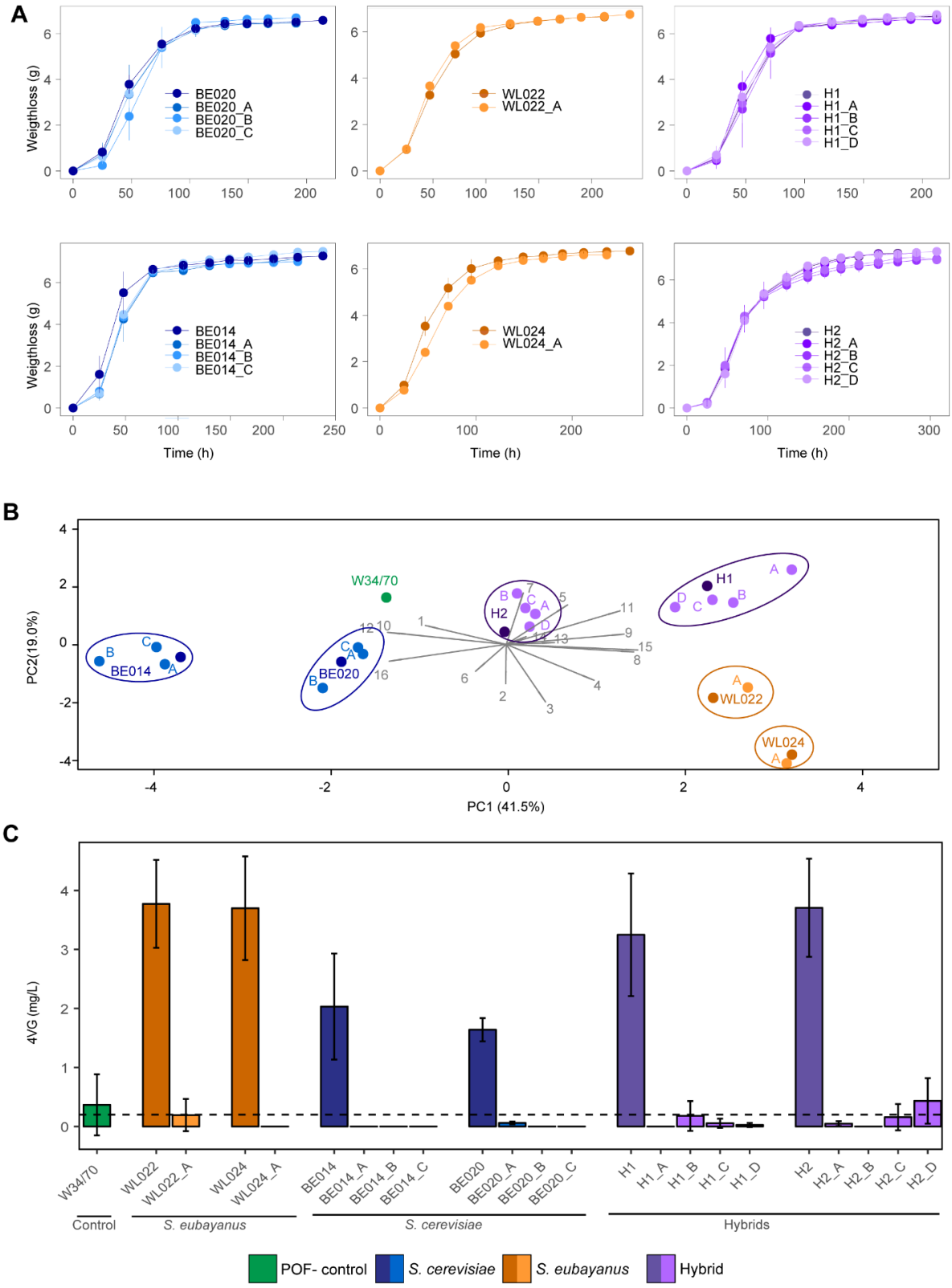

Figure 31: Gene edited variants behave similar as their parental strains in lab scale beer fermentations, except for their POF phenotype. *(A), fermentation profiles based on daily weight measurements of BE020, BE014, WL022, WL024, H1 and H2 with their respective gene edited mutants. (B) Principle Component Analysis (PCA) based on the production of 16 different metabolites, describing 60.5% of the total variability. Eigenvectors of the different variables are depicted with numbers ranging*

from one to 16. 1 = Ethanol, 2 = glycerol, 3 = SO2, 4 = acetaldehyde, 5 = ethyl acetate, 6 = ethyl propionate, 7 = propyl acetate, 8 = isoamyl alcohol, 9 = isobutyl acetate, 10 = ethyl butyrate, 11 = isoamyl acetate, 12 = ethyl hexanoate, 13 = phenetyl alcohol, 14 = ethyl octanoate, 15 = phenetyl acetate and 16= ethyl decanoate. (C) 4VG production (mg.L $^{-1}$) of W34/70 (green), S. eubayanus WL022 and WL024 (dark orange) with their respective gene edited variants (light orange), *S. cerevisiae* BE014 and BE020 (dark blue) with their respective gene edited variants (light blue), as well as for artificial interspecific hybrids H1 and H2 (dark purple) with their gene edited variants (light purple). Error bars represent the standard deviation of two biological replicates. Dotted line represents the flavour threshold of 4VG in beer (0.3mg.L^{-1}), as reported in (167).

4.6 Discussion

Today's beer market is characterized by an increased demand for nice products and diversification (30). These market forces have led to an increased interest into novel beer yeasts that can impart new aroma's. However, some of the most interesting strains are characterized by the production of the undesirable aroma compound 4VG. Here we describe a new CRISPR-based gene editing strategy that allows to specifically modify 4VG production in various yeasts, including feral *Saccharomyces cerevisiae* isolates, non-*cerevisiae* strains and interspecific hybrids.

Compared to previous approaches aimed at modifying industrially-relevant phenotypes in yeast (73, 172, 174), this study introduces a naturally-occurring point mutation, rather than deleting the gene of interest, thereby generating cisgenic variants that are likely exempted from GM regulations within countries like Argentina, USA, Japan and Brazil (45, 72, 179).

Gene editing efficiency ranged from 100 percent for lab strains and diploid industrial *S. cerevisiae* strains to 10% for more complex, poly- and euploid industrial *S. cerevisiae* yeasts. We also report the first CRISPR-based engineering of *S. eubayanus*, which was also highly efficient (100%). The reported gene editing efficiencies are in line with previous reported efficiencies in industrial relevant *S. cerevisiae* strains when performing CRISPR-based gene deletions (1.3% up to 100% (73, 159, 172)).

Although CRISPR-based editing was previously reported to be an efficient route to modify *S. pastorianus* (174), the Cas9-induced double-stranded DNA break caused an unexpected and undesirable partial chromosome loss (99). Off-target activity of CRISPR genome editing in haploid or homozygous *Saccharomyces* yeasts has been shown to be very rare (159). More recently however, allele specific gene editing in artificial *S. cerevisiae* X *S. eubayanus* hybrids was reported to cause loss of heterozygosity (LOH). Specifically, the induced double-stranded

break in the *S. eubayanus* derived locus was not repaired by the delivered repair template via homology-directed repair, but rather repaired via loss of heterozygosity (175). Although further research is needed, the fact that *FDC1* is located in the subtelomeric region in *S. cerevisiae* (Chr IV) and *S. euybayanus* (Chr13) could favor partial chromosome loss over repair via LOH. One way to reduce such unwanted structural rearrangements is by designing the repair template in such a way that the PAM site is inactivated (73). This prevents continued cutting of the site by the Cas9 endonuclease after successful introduction of the desired mutation (35). However, this strategy is not ideal for editing organisms that are targeted for food production because in most cases, the resulting mutant would not be cisgenic anymore and thus subjected to normal GM laws. We therefore modified the gene-editing protocol by increasing the amount of repair template and reducing the CRISPR cas9 endonuclease activity, which seemed to reduce the unwanted genomic rearrangements and increased repair via the repair template. The proposed technique showed a seven to 12% efficiency in specifically introducing a single SNP in the subtelomeric *S. eubayanus* derived *FDC1* gene in novel generated interspecific hybrids.

Overall, some of the generated POF^- variants show great potential for industrial application, as their unique aroma profiles are no longer masked by the 4VG. Additionally, the CRISPR gene editing strategy described in our study offers a general tool for tuning the characteristics of various aneuploid and non-Saccharomyces yeasts. Specifically, the combination of our gene editing protocol with the ever-increasing number of identified quantitative trait loci (QTL) represents a formidable opportunity to obtain superior industrial yeasts through gene editing (93).

5

General conclusions and future perspectives

5.1 General conclusion

Historically, the emergence of industrial man-made environments, like beer fermentations, challenged microorganisms with completely novel ecological niches, and called for adaptation of existing microbes in order to be colonized (54). Recent whole genome sequencing showed that interspecific hybridization is an important and common route towards diversification and adaptation to such novel niches. Indeed, interspecific hybridization provides a rapid way to combine distinct phenotypes from established populations and, combined with its characteristically genetic plasticity, can lead to a unique combination of phenotypes, enabling interspecific hybrids to thrive in a new environment and outcompete their parental species. For example, hybridisation of the sunflowers *Helianthus annus* and *Heliantus petiolaris* gave rise to three novel species capable of colonizing previously untapped environments. More precisely, the resulting novel interspecific hybrid species are able to combine the resistance towards high salinity and draught of both parental species, allowing them to respectively thrive in sand dunes, basin deserts, and desert salt marshes, all of which are extreme environments that are inaccessible to the parental species (142). Recently, next generation sequencing revealed the presence of several interspecific hybrid yeasts within the *Saccharomyces* yeast clade (23, 60, 139). While some hybrids have occasionally been isolated from natural habitats, most isolates were obtained from man-made industrial environments (24, 40). The best-known and most-studied example of such interspecific hybrid is the lager yeast *S. pastorianus*. *S. pastorianus* is shown to be the result of an interspecific hybridization between *S. cerevisiae* and *S. eubayanus* and combines the good fermentation capacity of *S. cerevisiae* with the cold tolerance of *S. eubayanus*, enabling it to thrive in cold temperature driven industrial beer fermentations (89, 116, 178, 182).

This rapid way of combining phenotypes of different species into one organism sparked researchers worldwide to generate novel interspecific hybrids within the lab to answer current challengers within the fermentation industry. Indeed, after a long period of consolidation and homogenization, the past years have brought a remarkable increase in the demand for specialty beer and a decrease in the consumption of traditional beer-styles like lager beers, turning the global beer market into a niche market where product diversification has become pivotal. Driven by a changing consumer demand for more product variety and more ‘local’ products and the increasing awareness of the negative effects of alcohol consumption,

brewers are pushed towards product innovation and differentiation in order to reinsure their competitive market position.

Different routes towards product differentiation exist. For example, brewers can choose from an ever increasing variety of specialty malt, hops and spices, introducing novel aromas and flavours and potentially increase the aromatic complexity of existing beers. Another tool skilled brewers are provided with, is tweaking brewing- and fermentation-parameters. For example, fermenting at higher temperatures generally increases the amount of aromatic esters produced by yeast (84). Complementary to the above, changing the yeast used during fermentation shows great potential. Not only does yeast produce a wide variety of secondary metabolites, that differs significantly from aromas that can be introduced by malt, hops or spices (170), it also allows brewers to differentiate their beer without the need for dramatic and costly changes to their production process.

Within this thesis, a spore-to-spore breeding technique was applied to cross six different and carefully selected *S. cerevisiae* yeasts with two wild *S. eubayanus* yeasts in order to broaden the lager beer aromatic diversity and to fill the existing gap between highly drinkable lager beer and aroma rich ales. A representative set of 31 novel interspecific yeast hybrids were phenotyped for their temperature tolerance and fermentation capacity as well as their aroma production in lab scale fermentation trails. Four selected hybrids were later on tested in pilot fermentation trails, revealing the industrial relevance of at least one of the generated hybrids, H29, for the production of a novel, more aroma-rich lager beer. Recently, lab-generated interspecific hybrids between *S. cervisiae* and cold tolerant *Saccharomyces* species like *S. uvarum* (117, 147, 148), *S. eubayanus* (81, 98, 117)(66, 80, 105), *S. kudriavzevii* (4, 15) or *S. arboricola* (117) were generated and proved to be highly successful in cold temperature driven beer, wine and cider fermentations. Indeed, the novel generated interspecific hybrids were, like *S. pastorianus*, able to combine the good fermentation capacity of *S. cerevisiae* with the cold tolerance of the non-*cerevisiae* yeasts, allowing them to perform better in cold temperature driven fermentations in comparison to both parental species.

Because of its industrial relevance, multiple studies focused on unravelling the temperature tolerance of *Saccharomyces* yeasts (122). For example, it has been shown that an increased glycerol accumulation and production of cytosolic acetaldehyde, potentially allowing to compensate for a temperature-induced redox imbalance, is crucial for the cold tolerance of *S.*

kudriavzevii. Also, it has been shown that an increased amount of unsaturated fatty acids and lowered concentrations of ergosterol within the cell membrane of *S. cerevisiae* X *S. eubayanus* hybrids is beneficial for their cold tolerance (81). More recently however, it was shown that the origin of mitochondrial DNA plays an important role in temperature tolerance of interspecific yeast hybrids (7, 67, 88). Indeed, artificially-constructed interspecific *S. cerevisiae* x *S. eubayanus* or *S. cerevisiae* x *S. uvarum* hybrids containing only *cerevisiae*- or non-*cerevisiae*-derived mitochondria, showed a significantly different temperature tolerance. When interspecific hybrid yeasts contain only *cerevisiae*-derived mitochondria, they show a temperature tolerance more similar to traditional ale brewing yeasts, whereas '*eubayanus*- or *uvarum*-derived mitochondria' containing hybrids show a better tolerance towards cold temperatures. Interestingly, within this thesis, the generated interspecific hybrids showed a broadened temperature tolerance compared to both their parental species, as they were able to both grow at low (4°C-16°C) and high (30°C-37°C) temperatures. Although further research is needed, a possible cause for the observed broadened temperature tolerance could be that the applied genetic stabilization procedure within this thesis favoured the generated interspecific hybrid yeasts to retain both *cerevisiae*- and *eubayanus*-derived mitochondria, rather than the previously observed uniparental inheritance of mitochondria by novel interspecific hybrids (13).

Generated interspecific hybrids are unable to form viable spores due to postzygotic reproductive barriers (<1% viable meiotic spores). Currently, there are three major hypotheses explaining this reproductive isolation (39). The first potential cause is the occurrence of gross chromosomal rearrangements within the two parental species. This has proven to be the case for some *S. cerevisiae* yeasts, where segregating translocations and inversions lead to spore inviability. Interestingly, this does not seem to be the major reason of speciation within the *Saccharomyces* clade, as clear signs of gene flow within yeast populations marked with gross chromosomal rearrangements have been observed (92). Secondly, so called Bateson–Dobzhansky–Müller incompatibilities can lead to hybrid sterility. This happens when in a hybrid genome, certain combinations of alleles are formed, which render the organism inviable. Within the *Saccharomyces* clade, only a hand full examples of such a speciation genes are found. For example, it was shown that *APE2*, located on chromosome 13 of *S. bayanus* is incompatible with *S. cerevisiae* mitochondria, causing spore

inviability in F1 hybrids (85). The third an most plausible mechanism causing interspecific hybrid sterility within the entire *Saccharomyces* clade is a combination of simple sequence divergence and mismatch repair (39). Here, multiple sequence differences between parental strains in the intermediates of homologous recombination, which are required for crossing over, chiasmata formation and proper chromosome segregation, are recognized by the mismatch-repair system. Rather than being "repaired" to one of the parental sequences, this large number of mismatches leads to abortion of the intermediate, resulting in random segregation of chromosomes and the non-occurrence of recombination (22).

The observed sexual sterility of (novel generated) interspecific yeast hybrids confines them to a vegetative life style. Even though sexual sterility is preferred for industrial yeasts (as this improves the genetic, and thus phenotypic stability when reused for different rounds of fermentation), it does hamper the use of more traditional, breeding-dependent strain improvement strategies to further fine-tune these novel yeasts (156). Luckily, molecular biotechnology offers a wide range of tools, allowing researchers to overcome or by-pass this interspecific hybrid sterility. The most promising, but maybe most controversial technique at the moment is CRISPR-Cas9-based gene editing (99). The use of this technique in (micro) biology is prospering, as it allows researchers to very precisely alter the genome of their favourite microbe or organism, without leaving any genetic markers or scars behind. In combination with the increasing amount of studies trying to unravel the genetics behind different industrial relevant phenotypes (93), CRISPR-Cas9-based gene editing can cause a big leap forward into yeast strain improvement. Moreover, this technique allows researchers to go beyond what nature has to offer, as it can be used to introduce completely novel pathways into the organism of choice (159, 179). Therefore, the recent view of the European Union regarding the classification of CRISPR-Cas9-gene edited organisms as genetically modified is very regretful, and feels like a missed opportunity to fulfil the role of the EU as one of the major drivers in biotechnology (159, 173). Even more so when the technique is used to generate cisgenic variants, meaning the introduction of genetic material of closely related organisms, which could, in theory, have been obtained via traditional breeding or random mutagenesis (173). Within this thesis, cisgenic POF^- variants of genetically complex yeasts were generated, which highly improve their industrial applicability. The optimized CRISPR-cas9-based gene editing strategy allows to use this technique in genetically complex pure, and

interspecific yeast species, something that has been shown to be very difficult (175). Indeed, for the first time, CRISPR-Cas9-based gene editing has been applied on artificial generated interspecific hybrids without introducing previously reported of target activity or the introduction of loss of heterozygosity (175).

The emerging trend to generate and explore new yeast variants in the fermentation industry triggered the development of several strategies to generate new yeast variants on a large scale (reviewed in (156)). Using these strategies, hundreds or even thousands of different yeast variants can be developed rapidly. Taken together these emerging trends have as a result that not the development, but rather the screening of these yeasts for specific industrially-relevant attributes has become a major bottleneck. Therefore, there is a strong need for high-throughput assays, to quickly assess industrially-relevant characteristics at a low cost. The developed POF screening assay within this thesis is a good example of such a screening tool, as it allows the phenotyping of hundreds of yeasts in parallel with only one hour of hands-on-labour.

5.2 Future perspectives

Within this thesis, different novel yeasts were generated and different techniques were developed, which still show great potential for further research.

First off all, only a small but representative subset of the generated interspecific hybrids between selected *S. cerevisiae* and *S. eubayanus* yeasts were phenotyped in great detail and therefore reported upon within this thesis. The remaining generated interspecific hybrids (Table S5), which resulted from crossing *S. cerevisiae* yeasts with *S. eubayanus, S. kudriavzevii* or *S. mikatae*, could potentially still hold great potential for industrial application. The most promising hybrids could potentially be tested on a larger scale in our novel 5hL pilot brewery, allowing us to better asses their industrial applicability. Furthermore, it would be interesting to investigate our newly generated interspecific hybrids in Northern wine and cider production (characteristically at lower temperatures), since the use of such hybrids has been shown to be very promising in these processes (61, 98).

Secondly, besides the industrial application of the generated interspecific hybrids, a more fundamental study, using the two large pools of generated interspecific hybrids, each resulting from crossing the same two parental strains, could yield further insights in for example the

cold-tolerance of the interspecific hybrids. Some attempts in understanding cold-tolerance in non-*cerevisiae* yeasts and interspecific *Saccharomyces* yeast hybrids are already made, but the exact underlying mechanism still remains to be discovered (7, 67, 88, 122, 166).

Thirdly, the optimized CRISPR-Cas9 based gene editing strategy can be used as a tool to alter other industrially-relevant phenotypes (besides their POF phenotype) in industrial yeast strains. For example, the CRISPR-Cas9-based gene editing strategy was recently used to introduce a set of different SNPs in both ATF1 and IMP1, reducing the ester production of industrial *S. cerevisiae* yeasts (patent application PCT/EP2018/052084 (171)). Moreover, the designed protocol is currently used in multiple different fundamental studies within our lab.

Finally, the striking observation that beer yeasts selectively lost the ability to form 4VG, whereas all wild yeasts retained this phenotype as a possible detoxification mechanism for hydroxycinamic acids, needs further investigation (54). A possible theory is that being POF^- has a competitive advantage in the brewing environment, allowing POF^- yeasts to dominate beer fermentations, rather than being the consequence of human/brewer's interventions and selection. This theory is strengthened by the fact that different lines of brewing yeast independently from each other evolved from being POF^+ to POF^- (54). First insights in possible fitness effects of being POF^- could be investigated by performing direct competition assays of the generated POF^- cisgenic *S. cerevisiae* yeasts with their respective parental strains.

6

Appendices

6.1 Supplemental tables

Table S1 overview aroma and ethanol production from lab scale lager beer fermentation tests. DNA content is represented as times the average DNA content of the haploid reference yeasts ("/" means not measured). Quantified yeast-related aroma compounds are represented as concentrations ($mg.l^{-1}$) and the measured final ethanol content as volume percentages. Lastly, the used score legend for flavours during sensory analysis was: VS = very slightly; S = slightly; V = very; N =neutral; SW = sweet; FR = fruity; FL = floral; SULF = sulphury; SOLV = solvent-like; FRESH = fresh.

	DNA content	Acetaldehyde ($mg.l^{-1}$)	Ethyl acetate ($mg.l^{-1}$)	Ethyl propionate ($mg.l^{-1}$)	Isobutyl acetate ($mg.l^{-1}$)	Ethyl Butyrate ($mg.l^{-1}$)	Propanol ($mg.l^{-1}$)	Isobutanol ($mg.l^{-1}$)	Isoamyl acetate ($mg.l^{-1}$)	Butanol ($mg.l^{-1}$)	Isoamyl alcohol ($mg.l^{-1}$)	Phenyl ethyl acetate ($mg.l^{-1}$)	Phenyl ethanol ($mg.l^{-1}$)	ethanol ($\%vv^{-1}$)	Sensoryanalysis
H1	1,67	22.36	13.78	0.25	0.09	0.11	6.79	12.08	1.12	0.64	48.78	0.70	6.97	4.78	SW
H2	1,39	23.18	11.88	0.22	0.07	0.10	5.72	9.01	1.13	0.52	38.21	0.81	9.00	4.54	SW
H3	1,69	22.64	14.77	0.33	0.07	0.08	8.85	14.19	0.91	0.75	71.91	0.88	12.09	5.56	SULF
H4	1,64	29.30	13.80	0.28	0.08	0.12	7.07	10.19	1.20	0.69	46.94	1.02	11.87	3.74	VSSW
H5	1,75	20.94	20.05	0.43	0.08	0.13	6.70	10.56	1.66	0.71	52.26	1.22	17.85	3.88	SW,FRESH
H6	2,07	19.59	13.86	0.28	0.07	0.10	6.39	9.91	1.18	0.00	37.85	0.76	9.78	4.48	N
H7	1,72	19.33	15.00	0.26	0.10	0.09	7.17	12.93	1.25	0.76	56.07	1.28	14.01	3.78	SSW
H8	1,91	19.56	9.02	0.29	0.06	0.12	6.62	11.97	0.77	0.86	53.93	0.75	13.25	3.76	FR,SW
H9	1,59	19.84	8.60	0.24	0.08	0.06	6.49	14.16	1.07	0.74	57.72	1.09	32.00	2.96	SW,SFL
H10	1,85	23.68	12.18	0.32	0.06	0.08	6.91	10.90	0.89	0.68	46.49	0.87	10.07	3.96	SW,SSLUF
H11	1,68	14.67	10.09	0.30	0.05	0.06	5.90	8.78	0.68	0.67	41.59	0.91	12.02	4.62	N, SSULF
H12	2,91	6.97	14.89	0.16	0.07	0.11	5.62	9.27	1.06	0.19	36.12	0.74	8.79	5.30	SW,FR
H13	3,62	19.69	15.35	0.28	0.08	0.18	6.25	10.98	1.23	0.26	39.67	0.86	11.18	5.50	SSW,SFL

H14	2,36	9.21	19.84	0.48	0.09	0.16	9.92	14.82	1.54	1.32	75.96	1.10	27.37	4.70	SW, SSULF
H15	3,15	16.83	22.26	0.25	0.07	0.19	9.32	8.27	1.43	1.02	44.58	0.55	5.89	6.20	N,SULF
H16	2,84	14.93	24.73	0.34	0.09	0.14	8.83	10.91	1.44	0.84	48.40	0.67	6.20	5.18	SW,FL
H17	3,05	14.03	26.42	0.36	0.09	0.22	7.76	8.81	1.82	0.85	37.72	0.89	6.96	5.72	SSW,SFL
H18	3,43	20.91	18.49	0.29	0.09	0.21	7.94	9.45	1.45	1.03	44.23	0.69	7.92	5.32	N,SSULF
H19	3,10	16.76	15.10	0.24	0.07	0.10	6.14	8.19	1.18	0.79	39.79	0.54	6.48	5.16	SULF
H20	3,12	24.82	23.84	0.30	0.09	0.14	6.64	7.86	1.50	0.84	28.62	0.68	4.67	4.12	N,SSW
H21	3,56	24.50	14.92	0.16	0.06	0.14	7.34	8.18	1.11	0.89	40.04	0.62	7.38	4.50	SW,FL
H22	2,96	8.62	24.32	0.34	0.08	0.22	6.51	6.66	1.55	0.66	32.29	0.74	6.78	4.72	(S)FR
H23	2,85	19.34	19.61	0.39	0.06	0.14	8.20	8.64	1.13	1.13	44.99	0.67	7.30	5.32	SSW,SFR
H24	3,20	34.28	19.60	0.20	0.08	0.17	7.03	7.64	1.43	0.75	39.28	0.96	10.07	5.96	N, FR
H25	2,77	24.62	17.58	0.20	0.05	0.31	8.10	8.06	0.81	1.05	46.13	0.72	7.07	4.72	SSW,SFR
H26	3,01	23.16	14.58	0.53	0.05	0.25	6.36	7.00	1.01	1.03	41.61	0.80	7.95	5.08	SSW,FL
H27	3,03	16.59	14.28	0.26	0.06	0.08	5.89	7.61	0.99	0.76	39.20	0.80	7.89	4.28	N
H28	2,11	8.70	15.45	0.35	0.07	0.09	6.33	8.26	1.41	0.23	37.67	1.18	10.73	4.08	SW,FR
H29	2,58	14.24	26.32	0.46	0.14	0.19	9.38	12.96	2.66	0.86	73.77	2.15	41.02	6.12	FR, SW, FRESH
H30	2,24	22.63	24.31	0.40	0.10	0.14	7.01	7.72	1.68	0.77	34.97	1.12	10.18	4.76	FR, SW, FRESH
H31	3,23	22.50	28.11	0.36	0.12	0.20	5.97	8.11	2.40	0.86	38.41	1.31	9.83	4.78	FR, SW, FRESH
Y134	2,94	10.71	12.47	0.19	0.05	0.12	7.90	7.69	0.94	0.23	32.44	0.76	14.11	4.40	SFL,SSW, SOLV
Y184	1,77	20.21	7.61	0.16	0.05	0.04	7.54	11.65	0.66	0.55	40.77	0.70	31.83	3.10	SW,VFR
Y397	3,77	4.24	18.57	0.41	0.08	0.10	10.24	12.19	1.51	1.04	72.08	1.34	16.47	5.00	VFR,SSW
Y377	3,72	3.91	17.49	0.23	0.07	0.21	7.35	8.85	1.39	0.75	50.21	0.57	8.21	4.52	VSW

Y245	4,00	3.89	16.98	0.24	0.07	0.18	7.44	6.85	1.24	0.77	32.84	0.59	7.69	4.48	VFR,FL
Y470	1,55	13.42	7.30	0.16	0.05	0.04	6.13	9.08	0.64	0.62	36.76	0.58	14.16	2.92	SF,SSW
Y565	2,03	7.77	5.66	0.11	0.06	0.04	4.81	12.37	0.64	0.68	48.13	0.80	13.09	2.76	SSULF
Y567	1,59	16.58	7.36	0.20	0.07	0.05	5.68	14.96	0.69	1.01	56.04	0.87	17.55	2.38	SULF
GSY132	2,52	7.01	9.26	0.36	0.05	0.07	5.70	10.59	0.74	2.80	39.31	0.74	13.85	4.20	N
GSY 501	3,61	9.21	7.68	0.43	0.03	0.18	6.47	8.05	0.36	1.00	38.08	0.53	16.94	4.56	VSW,F,FR
GSY134	/	28.20	5.23	0.30	N.D.	0.06	4.82	6.30	0.36	0.16	35.33	0.74	18.17	3.50	VSSW, N
GSY133	/	8.37	5.84	0.39	0.03	0.12	5.28	7.28	0.35	0.16	30.04	0.49	22.77	4.48	N
GSY129	/	9.37	11.19	0.33	0.05	0.11	5.28	10.23	0.89	N.D.	38.62	0.61	13.04	4.46	VSSULF, N
GSY131	/	18.23	8.59	0.32	N.D.	0.10	4.97	5.57	0.51	0.20	34.98	0.64	22.17	6.10	VSSULF,N
KV2062	/	8.75	9.23	0.28	0.05	0.16	5.75	12.06	0.69	N.D.	43.82	0.79	8.94	5.56	N, VSSW
GSY137	/	13.20	7.33	0.32	N.D.	0.08	4.99	5.93	0.47	0.22	36.34	0.40	8.03	4.82	VSSULF, N
Y449	/	11.69	25.07	0.38	0.07	0.15	5.95	6.66	1.39	0.26	26.69	0.63	4.96	5.98	FR
Y473	/	11.73	24.78	0.57	0.09	0.09	9.09	11.14	1.38	0.90	40.28	1.55	12.59	4.70	N
Y453	/	7.12	19.36	0.46	0.06	0.10	8.60	8.35	1.19	0.55	35.37	1.20	9.30	5.96	VSSULF,N
Y454	/	9.46	21.32	0.41	0.09	0.09	7.61	9.38	1.50	0.74	37.62	1.46	9.33	5.98	N
GSY516	/	11.49	18.07	0.26	0.07	0.11	7.82	8.26	0.98	0.22	29.31	0.53	8.69	5.24	VSSULF,VSSW
GSY135	/	11.47	15.43	0.54	0.05	0.10	9.46	6.57	1.01	0.36	30.76	1.12	27.47	3.92	VSSW
Y472	/	11.78	24.60	0.48	0.08	0.12	6.71	6.13	1.28	0.22	24.54	0.90	5.63	6.94	N
GSY515	/	11.52	26.40	0.52	0.11	0.08	7.58	11.11	1.83	0.59	37.91	1.51	6.80	6.08	SW,FR
Y5	/	7.19	17.04	0.66	0.06	0.09	10.17	10.39	1.12	0.87	44.72	0.86	9.09	4.96	N, SFL

Table S2: obtained results Kruskal-wallis test and pair wise post hoc Dunn tests for comparison growth capacity of the generated interspecific hybrids with parental strains (Kruskal-Wallis test P-value or Benjamini- Hochberg corrected p-value < 0.05: (*) or < 0.01 : (**))

	Kruskal-wallis test		Dunn test with bh-corrected p-values								
			S. cerevisiae - hybrid			*S. eubayanus* - hybrid			*S. eubayanus* - *S. cerevisiae*		
	Chi-squared	P-value	Z	P-value	corrected P-value	Z	P-value	corrected P-value	Z	P-value	corrected P-value
4°C (15days)	16.12(**)	0	3.25 (**)	0.0006	0.0009	-2.02 (*)	0.0215	0.0215	-3.58 (**)	0.0002	0.0005
8°C (5days)	13.48 (**)	0	2.82 (**)	0.0024	0.0036	-2.06 (*)	0.0195	0.0195	-3.38 (**)	0.0004	0.0011
10°C (5 days)	10.98 (**)	0	2.39 (*)	0.0084	0.0125	-2.04 (*)	0.0205	0.0205	-3.13 (**)	0.0009	0.0026
16°C (2days)	8.62 (**)	0.01	2.51 (*)	0.0061	0.0182	-1.27	0.1017	0.1017	-2.5 (**)	0.0061	0.0091
30°C (2days)	6.05 (*)	0.05	0.89	0.1858	0.1858	2.37 (*)	0.0089	0.0267	1.63	0.0516	0.0775
37°C (2days)	11.82 (**)	0	-2.50 (**)	0.0061	0.0092	2.1 (*)	0.0180	0.0180	3.24 (**)	0.0006	0.0018
41°C (2 days)	27.31 (**)	0	-5.17 (**)	0.0000	0.0000	0.26	0.3974	0.3974	3.05 (**)	0.0011	0.0017

Table S3: obtained results Kruskal-wallis test and pair wise post hoc Dunn tests for comparison aroma production of the generated interspecific hybrids with Saaz- and Frohberg- type reference *S. pastoriuanus* strains (Kruskal-Wallis test P-value or Benjamini-Hochberg corrected p-value < 0.05: (*) or < 0.01 : (**))

	Kruskal -wallis test		Dunn test with bh -corrected p-values								
			hybrid - Frohberg			hybrid - Saaz			Saaz - Frohberg		
	Chi-squared	P-value	Z	P-value	corrected P-value	Z	P-value	corrected P-value	Z	P-value	corrected P-value
acetaldehyde	15.39 (**)	0	-3.69 (**)	0.0001	0.0003	-2.08	0.0187	0.0281	-0.96	0.1694	0.1694
ethyl acetate	17.72 (**)	0	1.26	0.1040	0.1040	-3.66 (**)	0.0001	0.0002	4.04 (**)	0.0000	0.0001
ethyl propionate	11.54 (**)	0	3.39 (**)	0.0004	0.0011	0.99	0.1614	0.1614	1.66	0.0486	0.0729
isobutyl acetate	16.99 (**)	0	-0.37	0.3554	0.3554	-4.09 (**)	0.0000	0.0001	3.20 (**)	0.0007	0.0010
ethyl butyrate	6.52 (*)	0.04	-2.50 (**)	0.0062	0.0186	-1.03	0.1516	0.2274	-0.97	0.1657	0.1657
Propanol	14.89 (**)	0	1.01	0.1561	0.1561	-3.43 (**)	0.0003	0.0005	3.65	0.0001	0.0004
isobutanol	4.34	0.11	-0.32	0.3757	0.3757	-2.08	0.0188	0.0563	1.53	0.0627	0.0941
isoamyl acetate	16.22 (**)	0	-0.3	0.3822	0.3822	-3.99 (**)	0.0000	0.0001	3.17 (**)	0.0008	0.0012
butanol	9.94 (*)	0.01	-0.99	0.1618	0.1618	-3.14 (**)	0.0009	0.0026	1.93	0.0265	0.0398
isoamyl alcohol	11.68 (**)	0	-2.85 (**)	0.0022	0.0065	-2.45 (**)	0.0072	0.0108	-0.03	0.4893	0.4893
phenyl ethyl acetate	11.17 (**)	0	1.22	0.1109	0.1109	-2.78 (**)	0.0027	0.0041	3.26 (**)	0.0006	0.0017
phenyl ethanol	4.03	0.13	0.17	0.4308	0.4308	1.99	0.0232	0.0695	-1.56	0.0590	0.0885

Table S4: Overview results sensory evaluation from pilot scale fermentation tests by professional tasting panel. Score legend for flavours: absent (/); very very slightly present (- - -); very slightly present (- -); slightly present (-); mildly present (- +); present (+); clearly present (++); predominant (+++).

		Y134	Y397	Y470	GSY132	GSY501	Y567	H4	H5	H15	H29
Aroma	**malt**	grainy (-)	/	grainy (- -)	/	/	/	grainy (+)	grainy (-)	grainy (-)	/
	hoppy	/	/	grassy (+)	/	/	/	/	/	/	/
	esters	/	fruity (banana/ pineapple) (++)	fruity (-)	/	fruity (pineapple) (++)	/	/	/	/	fruity (banana) (-)
	phenolic	/	/	/	/	/	/	smoke (+)	/	/	/
Flavour	**Malt**	/	/	grainy (- -)	grainy (- -)	/	/	grainy (+)	grainy (- +)	grainy (+)	/
	Hoppy	/	/	grassy (+)	/	/	/	/	/	/	/
	esters	fruity (apple/pear) (-)	fruity (banana/ pineapple) (++)	fruity (- -)	/	fruity (pineapple) (++)	/	/	/	/	fruity (banana) (+)
	phenolic	/	/	/	/	/	smoke (-)	smoke (+)	/	/	/

other		sulphur (- +)	sulphur (+)/onion (+)	/	maillard (- +)/oxidized (-)/ metallic (+)	/	sulphur (-)/ onion (+)	metallic (+)	SO2(-+)/eggs(-)/ rubber (-)	sulphur (- +)/ metallic (+)	sulphur (-)
taste/mouthfeel	**Bitterness intensity**	+	+	+	+	+	+	+	-+	+	+
	bitterness quality	+++	+	+++	- -	+ + +	+	- +	+ + +	+	+ + +
	sweet	-	-	- - -	- +	- -	- - -	-	- - -	- -	- -
	body	-	-	-	-	-	-	- +	- +	-	+
	astringency	-	- -	- - -	- +	- - -	- -	+ +	- -	- +	- - -
Overall	**appreciation**	+	- +	+	- -	+	-	- -	+	-	+
	complexity	- +	- +	--	-	- +	+ +	- +	- +	- +	+
	balance	+	- -	- +	- - -	+	- -	- - -	+	- - -	- +

Table S5: Overview of generated interspecific hybrids within this PhD thesis. A grey box indicate that no attempts were made to cross that particular combination of strains

S. cerevisiae	*S. eubayanus*		*S.mikatae*		*S.kudriavzevii*
	WL022 (Y565)	WL024 (Y567)	NCYC 2970	NCYC2888	NCYC 2889
BE006 (Y134)	11	101	5	3	2
BE011 (Y202)	1	0			
BE014 (Y245)	3	4			
BE023 (Y377)	4	3			
BE033 (Y470)	4	8			
WI009 (Y184)	4	12	2	2	2
SA003 (Y141)	3	1			
SP003 (Y146)	2	5			
BI003 (Y115)		107			
BE025 (Y397)	2	4			

Table S6: Statistical analysis of the weight loss measured during fermentation between gene edited variants and their respective WT (from time point 1 to end of the fermentation). P-values were obtained using ANOVA. All statistical analysis were conducted in R, with the multcomp package (* P-value < 0.05; ** P-value <0.01; *** P-values <0.001). "/" means fermentations were stopped before this time point.

	ANOVA					
P-values	**H1 vs gene edited H1 variants**	**H2 vs CRISPRH2**	**BE014 vs CRISPR BE014**	**BE020 vs CRISPR BE020**	**WL022 vs CRISPR WL022**	**WL024 vs CRISPR WL024**
T1	0.933	0.494	0.032	0.332	0.846	0.295
T2	0.900	0.953	0.071	0.331	0.069	0.068
T3	0.629	0.964	0.104	0.779	0.000***	0.129
T4	0.690	0.760	0.488	0.775	0.008**	0.216
T5	0.418	0.569	0.801	0.975	0.349	0.212
T6	0.973	0.309	0.378	0.593	0.839	0.179
T7	0.747	0.357	0.804	0.711	0.902	0.304
T8	0.783	0.349	0.790	0.677	1.000	0.268
T9	/	0.445	/	/	0.819	0.059
T10	/	0.246	/	/	/	/

Table S7: Statistical analysis of the phenotypic behavior of H1 compared to the H1 gene-edited variants. Column two represents the P-values obtained with ANOVA. Column three to twelve represent the obtained P-values of a post-hoc Tukey test. All statistical analyses were conducted in R, within the multcomp package (* P-value < 0.05; ** P-value <0.01; *** P-values <0.001).

	ANOVA	POSTHOC TUKEY									
P-values	H1 vs gene edited H1 variants	H1_A - H1	H1_B - H1	H1_C - H1	H1_D - H1	H1_B - H1_A	H1_C - H1_A	H1_D - H1_A	H1_C - H1_B	H1_D - H1_B	H1_D - H1_C
Ethanol	0.814	0.935	0.449	0.984	0.972	0.210	0.730	1.000	0.689	0.250	0.810
Glycerol	0.903	1.000	0.916	0.856	0.997	0.936	0.827	0.994	0.466	0.788	0.957
SO_2	0.927	0.645	0.965	0.968	0.822	0.922	0.371	0.235	0.739	0.517	0.989
Acetaldehyde	0.859	1.000	0.967	0.970	0.971	0.973	0.964	0.965	0.750	0.752	1.000
Ethyl acetate	0.059	0.923	0.444	0.321	0.476	0.824	0.664	0.856	0.997	1.000	0.993
Ethyl propionate	0.236	1.000	0.911	0.538	0.430	0.916	0.546	0.437	0.919	0.827	0.999
Propyl acetate	0.528	1.000	0.995	0.827	0.962	0.982	0.753	0.921	0.952	0.998	0.993
Isoamyl alcohol	0.0389 *	0.467	0.651	0.187	0.618	0.994	0.886	0.997	0.712	1.000	0.745
isobutyl.acetate	0.113	0.078	0.490	0.894	0.768	0.457	0.191	0.259	0.900	0.975	0.998
ethyl.butyrate	0.612	0.976	0.999	0.943	1.000	0.996	1.000	0.963	0.983	0.998	0.922
Isopentyl acetate	0.747	0.396	0.986	0.996	0.944	0.612	0.277	0.190	0.910	0.757	0.995
Ethyl hexanoate	0.437	0.202	0.901	1.000	1.000	0.479	0.239	0.167	0.949	0.830	0.996
Phenethyl alcohol	0.447	1.000	0.779	0.792	1.000	0.819	0.831	1.000	1.000	0.804	0.817
Ethyl octanoate	0.294	0.048	0.518	0.961	1.000	0.255	0.088	0.042	0.832	0.463	0.931
Phenethyl acetate	0.341	0.065	0.607	0.976	0.997	0.294	0.112	0.048	0.874	0.456	0.892
Ethyl decanoate	0.345	0.719	0.472	0.998	1.000	0.983	0.859	0.731	0.616	0.483	0.998
4VG	0.00***	0.005**	0.008**	0.005**	0.005**	0.965	1.000	1.000	0.970	0.982	1.000

Table S8: Statistical analysis of the phenotypic behavior of H2 compared to the H2 gene-edited variants. Column two represents the P-values obtained with ANOVA. Column three to twelve represent the obtained P-values of a post-hoc Tukey test. All statistical analyses were conducted in R, within the multcomp package (* P-value < 0.05; ** P-value <0.01; *** P-values <0.001).

	ANOVA	POSTHOC TUKEY									
P-values	**H2 vs gene edited H1 variants**	**H2_A - H2**	**H2_B - H2**	**H2_C - H2**	**H2_D - H2**	**H2_B - H2_A**	**H2_C - H2_A**	**H2_D - H2_A**	**H2_C - H2_B**	**H2_D - H2_B**	**H2_D - H2_C**
Ethanol	0.258	0.729	1.000	0.899	0.228	0.729	0.994	0.729	0.899	0.228	0.538
Glycerol	0.774	0.998	0.988	1.000	0.902	0.935	1.000	0.781	0.963	0.993	0.835
SO_2	0.572	0.999	0.426	0.984	0.971	0.516	0.948	0.994	0.258	0.707	0.808
Acetaldehyde	0.187	0.974	0.709	0.493	0.963	0.944	0.774	1.000	0.990	0.958	0.803
Ethyl acetate	0.600	1.000	0.815	0.974	0.999	0.883	0.992	0.995	0.984	0.713	0.926
Ethyl propionate	0.233	0.932	0.395	0.835	0.997	0.754	0.999	0.989	0.872	0.531	0.945
Propyl acetate	0.416	0.931	0.827	0.999	0.999	0.998	0.984	0.984	0.926	0.926	1.000
Isoamyl alcohol	0.412	0.896	1.000	0.960	0.832	0.860	0.999	1.000	0.938	0.791	0.994
isobutyl.acetate	0.725	0.999	0.990	1.000	0.999	0.999	1.000	1.000	0.993	1.000	0.999
ethyl.butyrate	0.176	0.927	0.561	0.854	0.945	0.918	0.999	1.000	0.968	0.895	0.998
Isopentyl acetate	0.558	0.999	0.887	0.990	1.000	0.959	1.000	1.000	0.988	0.908	0.994
Ethyl hexanoate	0.833	1.000	0.958	0.999	0.999	0.945	0.999	1.000	0.989	0.897	0.991
Phenethyl alcohol	0.229	0.733	1.000	0.828	0.522	0.774	0.999	0.991	0.864	0.562	0.964
Ethyl octanoate	0.361	0.998	0.998	0.825	0.834	1.000	0.935	0.941	0.934	0.940	1.000
Phenethyl acetate	0.258	0.951	0.892	0.894	0.963	1.000	1.000	1.000	1.000	0.999	0.999
Ethyl decanoate	0.351	0.935	0.997	0.494	0.726	0.822	0.855	0.983	0.368	0.572	0.987
4VG	0.000***	0.002**	0.001**	0.003**	0.003**	0.871	0.996	0.948	0.709	0.536	0.996

Table S9: Statistical analysis of the phenotypic behavior of BE014 compared to its gene-edited variants. Column two represents the P-values obtained with ANOVA. Column three to twelve represent the obtained P-values of a post-hoc Tukey test. All statistical analyses were conducted in R, within the multcomp package (* P-value < 0.05; ** P-value <0.01; *** P-values <0.001).

	ANOVA	POSTHOC TUKEY					
P-values	**BE014 vs f-gene edited BE014**	**BE014 vs BE014_A**	**BE014 vs BE014_B**	**BE014 vs BE014_C**	**BE014_A vs BE014_B**	**BE014_A vs BE014_C**	**BE014_B vs BE014_C**
Ethanol	0.138	0.741	0.790	0.284	1.000	0.712	0.661
Glycerol	0.199	0.399	0.217	0.997	0.915	0.479	0.262
SO_2	0.675	0.989	0.885	0.368	0.748	0.487	0.184
Acetaldehyde	0.054	0.316	0.340	0.637	1.000	0.862	0.893
Ethyl acetate	0.673	0.857	0.861	0.961	1.000	0.628	0.632
Ethyl propionate	0.206	0.910	0.063	0.695	0.111	0.961	0.172
Propyl acetate	0.630	0.741	0.979	0.996	0.909	0.632	0.928
Isoamyl alcohol	0.250	0.858	0.436	0.950	0.810	0.992	0.678
isobutyl.acetate	0.449	0.955	0.534	1.000	0.781	0.965	0.554
ethyl.butyrate	0.879	0.571	0.602	0.961	0.166	0.805	0.394
Isopentyl acetate	0.690	0.924	0.994	1.000	0.982	0.928	0.995
Ethyl hexanoate	0.961	0.973	1.000	0.983	0.981	0.866	0.976
Phenethyl alcohol	0.753	0.997	1.000	0.962	0.998	0.990	0.967
Ethyl octanoate	0.810	1.000	0.947	1.000	0.928	0.999	0.962
Phenethyl acetate	0.654	0.999	0.736	1.000	0.795	0.997	0.703
Ethyl decanoate	0.891	0.989	0.992	0.992	0.934	1.000	0.943
4VG	0.000***	0.029*	0.029*	0.027*	1.000	1.000	1.000

Table S10: Statistical analysis of the phenotypic behavior of BE020 compared to its gene-edited variants. Column two represents the P-values obtained with ANOVA. Column three to twelve represent the obtained P-values of a post-hoc Tukey test. All statistical analyses were conducted in R, within the multcomp package (* P-value < 0.05; ** P-value <0.01; *** P-values <0.001).

	ANOVA	POSTHOC TUKEY test					
P-values	**BE020 vs gene edited BE020**	**BE020 vs BE020_A**	**BE020 vs BE020_B**	**BE020 vs BE020_C**	**BE020_A vs BE020_B**	**BE020A vs BE020_C**	**BE020_B vs BE020_C**
Ethanol	0.560	1.000	0.158	0.987	0.159	0.987	0.117
Glycerol	0.085	0.413	0.647	0.605	0.951	0.970	1.000
SO_2	0.266	0.750	0.088	0.992	0.224	0.875	0.112
Acetaldehyde	0.221	0.674	0.751	0.912	0.998	0.949	0.980
Ethyl acetate	0.774	0.996	0.151	0.489	0.183	0.405	0.040
Ethyl propionate	0.557	0.888	0.010	0.945	0.007	0.998	0.007
Propyl acetate	0.896	0.983	0.477	0.857	0.342	0.969	0.225
Isoamyl alcohol	0.613	0.881	0.835	0.504	0.482	0.253	0.899
isobutyl.acetate	0.706	0.653	0.636	0.534	0.208	0.994	0.165
ethyl.butyrate	0.768	1.000	0.600	0.978	0.552	0.991	0.428
Isopentyl acetate	0.933	0.994	0.943	0.999	0.858	0.999	0.906
Ethyl hexanoate	0.400	0.754	0.418	0.996	0.886	0.650	0.344
Phenethyl alcohol	0.293	0.007	0.546	0.996	0.017	0.008	0.653
Ethyl octanoate	0.836	1.000	0.767	0.971	0.756	0.975	0.555
Phenethyl acetate	0.570	0.682	1.000	0.995	0.690	0.797	0.996
Ethyl decanoate	0.702	1.000	0.987	0.930	0.979	0.909	0.992
4VG	0.000***	0.000***	0.000***	0.000***	0.628	0.625	1.000

Table S11: Statistical analysis of the phenotypic behavior of WL022 compared to its gene-edited variant. Column two represents the P-values obtained with ANOVA. Column three to twelve represent the obtained P-values of a post-hoc Tukey test. All statistical analyses were conducted in R, within the multcomp package (* P-value < 0.05; ** P-value <0.01; *** P-values <0.001).

	ANOVA	POSTHOC-TUKEY
P-values	**WL022 vs gene edited WL022**	**WL022 vs WL022_A**
Ethanol	0.499	0.499
Glycerol	0.159	0.159
SO_2	0.246	0.246
Acetaldehyde	0.622	0.622
Ethyl acetate	0.154	0.154
Ethyl propionate	0.325	0.325
Propyl acetate	0.225	0.225
Isoamyl alcohol	0.389	0.389
isobutyl.acetate	0.248	0.248
ethyl.butyrate	0.192	0.192
Isopentyl acetate	0.159	0.159
Ethyl hexanoate	0.115	0.115
Phenethyl alcohol	0.390	0.390
Ethyl octanoate	0.866	0.866
Phenethyl acetate	0.087	0.087
Ethyl decanoate	0.636	0.636
4VG	0.025*	0.025*

Table S12: Statistical analysis of the phenotypic behavior of WL024 compared to its gene-edited variant. Column two represents the P-values obtained with ANOVA. Column three to twelve represent the obtained P-values of a post-hoc Tukey test. All statistical analyses were conducted in R, within the multcomp package (* P-value < 0.05; ** P-value <0.01; *** P-values <0.001).

	ANOVA	POSTHOC-TUKEY
P-values	WL024 vs gene edited WL024	WL024 vs WL024_A
Ethanol	0.228	0.228
Glycerol	0.054	0.054
SO_2	0.320	0.320
Acetaldehyde	0.969	0.969
Ethyl acetate	0.706	0.706
Ethyl propionate	0.495	0.495
Propyl acetate	0.229	0.229
Isoamyl alcohol	0.004**	0.004**
isobutyl.acetate	0.351	0.351
ethyl.butyrate	0.609	0.609
Isopentyl acetate	0.524	0.524
Ethyl hexanoate	0.266	0.266
Phenethyl alcohol	0.622	0.622
Ethyl octanoate	0.272	0.272
Phenethyl acetate	0.951	0.951
Ethyl decanoate	0.996	0.996
4VG	0.033*	0.033*

Table S13: overview aroma and ethanol production from lab scale lager beer fermentation tests. Quantified yeast-related aroma compounds are represented as concentrations ($mg.L^{-1}$), total weight loss as grams (g), ethanol production as volume percentage. Glycerol and SO_2 production are represented as concentrations ($g.L^{-1}$ and $mg.L^{-1}$ respectively). H_2S production capacity is qualitatively indicated (+,+-, -). Lastly, the used score legend for flavours during sensory analysis was: VS = very slightly; S = slightly; V = very; N =neutral; FR = fruity; POF = cloves, phenolic; FRESH = fresh.

Strain	total weight loss	ethanol	glycerol	SO2	acetaldehyde	ethyl acetate	ethyl propionate	propyl acetate	isoamyl alcohol	isobutyl acetate	ethyl butyrate	isopentyl acetate	ethyl hexanoate	phenethyl alcohol	ethyl octanoate	phenethyl acetate	ethyl decanoate	4VG	H_2S	Sensorial analysis
W34/70	8.00	6.97	3.13	1.54	9.07	32.62	0.83	0.05	206.23	0.16	0.18	1.93	0.33	95.01	0.86	0.74	0.04	0.26	+-	SFR/FRESH
BE014	7.14	6.11	3.29	1.73	0.25	13.36	0.35	0.01	69.70	0.03	0.07	0.73	0.10	12.11	0.23	0.03	0.53	2.03	-	VFR/SPOF
BE014_A	6.95	6.37	2.83	1.64	1.02	11.83	0.38	0.01	57.14	0.02	0.06	0.60	0.10	11.42	0.23	0.03	0.48	0.00	-	VFR
BE014_B	6.92	6.34	2.67	1.93	0.99	11.84	0.52	0.01	47.97	0.02	0.07	0.68	0.10	11.88	0.26	0.04	0.58	0.00	-	FR
BE014_C	7.43	6.64	3.24	1.20	0.72	14.28	0.40	0.01	59.87	0.03	0.06	0.73	0.10	10.54	0.23	0.03	0.48	0.00	-	FR
BE020	6.45	5.83	2.73	5.93	0.94	11.80	0.25	0.01	77.27	0.02	0.06	0.91	0.06	11.98	0.21	0.06	0.40	1.64	-	VSFR/POF
BE020_A	6.40	5.83	2.46	8.56	0.53	11.68	0.24	0.01	85.43	0.02	0.06	0.95	0.07	19.46	0.21	0.08	0.41	0.06	-	N
BE020_B	6.63	5.92	2.54	14.69	0.58	10.17	0.35	0.01	73.77	0.02	0.05	0.80	0.07	13.36	0.25	0.06	0.35	0.00	-	N
BE020_C	6.48	5.82	2.53	6.64	0.71	12.70	0.24	0.02	69.71	0.02	0.06	0.93	0.06	12.18	0.17	0.06	0.30	0.00	-	N
WL022	6.53	5.76	3.09	7.86	6.43	18.25	0.57	0.02	237.82	0.12	0.08	1.61	0.08	131.01	0.52	1.38	0.14	3.77	+	N/POF
WL022_A	6.56	5.79	3.22	9.53	8.82	21.72	0.45	0.03	238.53	0.14	0.09	1.93	0.11	115.40	0.58	1.59	0.26	0.12	+	SFR
WL024	6.64	5.71	3.20	19.61	13.66	21.05	1.39	0.01	227.34	0.19	0.09	1.68	0.11	129.05	0.87	1.48	0.16	3.70	+	SFR/POF
WL024_A	6.50	5.77	3.55	27.15	13.65	20.66	0.80	0.03	253.85	0.12	0.09	1.58	0.08	136.04	0.39	1.47	0.16	0.00	+	FR
H1	6.62	5.80	2.52	0.94	5.16	24.69	0.68	0.08	218.69	0.13	0.09	2.21	0.11	101.24	0.48	1.13	0.03	3.25	+-	SFR/SPOF
H1_A	6.49	5.85	2.56	1.06	5.14	23.68	0.69	0.08	199.72	0.17	0.09	2.82	0.14	107.37	1.06	1.61	0.18	0.00	+-	VFR
H1_B	6.59	5.69	2.94	0.99	3.45	22.34	0.82	0.07	206.35	0.15	0.09	2.39	0.12	155.46	0.74	1.35	0.23	0.10	+-	VFR
H1_C	6.63	5.77	2.03	0.89	7.84	23.29	1.00	0.07	202.99	0.15	0.09	2.27	0.12	165.15	0.63	1.30	0.08	0.00	+-	VFR
H1_D	6.64	5.84	2.37	0.86	6.52	21.24	0.94	0.07	195.16	0.14	0.09	1.91	0.10	101.99	0.45	1.05	0.04	0.00	+-	VFR
H2	7.13	6.23	2.69	1.23	4.62	24.64	0.92	0.04	223.01	0.12	0.11	1.71	0.16	104.91	0.67	0.75	0.21	3.71	+	FR/POF
H2_A	7.16	6.29	2.67	1.27	3.71	25.25	0.78	0.05	215.30	0.12	0.12	1.84	0.16	162.17	0.61	0.95	0.15	0.05	+	FR
H2_B	6.82	6.23	2.73	1.64	1.93	25.64	0.50	0.05	206.99	0.12	0.12	1.98	0.18	103.14	0.56	0.91	0.22	0.00	+	SFR
H2_C	6.99	6.27	2.68	1.12	1.44	25.38	0.70	0.04	209.39	0.11	0.12	1.85	0.16	147.83	0.46	0.94	0.07	0.15	+	FR
H2_D	7.18	6.36	2.76	1.36	3.84	25.28	0.90	0.05	223.89	0.13	0.13	1.86	0.16	199.46	0.51	1.00	0.12	0.43	++	FR

Table S14: overview used primers

Primer	Sequence (5' to 3')	use
SS_FWSC	AGAATTGCCCATCATCTGGG	*S. cerevisiae* specific forward sanger sequencing primer
SS_RVSC	ACCTTCAGGAATTGGCATGG	*S. cerevisiae* specific reverse sanger sequencing primer
SS_FWSE	GAATTGCTCATCATCTCGGG	*S. eubayanus* specific forward sanger sequencing primer
SS_RVSE	TCGCCCAAAATTGCACCGAT	*S. euba0yanus* specific reverse sanger sequencing primer
RT_FWSC	ATACATCTACAAAGCCTGCCAACACCATATCTACATGTTTCAGACGGTGGCAAGTACTTA	*S. cerevisiae* specific forward primer for the generation of the repair template
RT_RVSC	TTTTATCTGGAGTTTGAAGAATCCACATTCCGTACGTTTATAAGTACTTGCCACCGTCTG	*S. cerevisiae* specific reverse primer for the generation of the repair template
RT_FWSE	GTTCATTTAGATAGTCTTCCAGCACCATACTTGCATACTTCTGATGGTGGCAAGTATTTG	*S. eubayanus* specific forward primer for the generation of the repair template
RT_RVSE	TCTTATCTGGAGTTTGAAGAATCCACATTCCGTACGTTTACAAATACTTGCCACCATCAG	*S. eubayanus* specific reverse primer for the generation of the repair template
FW1	CCCTCTTTCTCTTTGCTTTC	See FIG S2
RV1	AGTAGAGAGGGCATAGATCG	See FIG S2
FW2	GAATTGCTCATCATCTCGGG	See FIG S2
RV2	TTGCCACCATCAGAAGTATG	See FIG S2
FW3	GGAAGACGTATGGCTACAAG	See FIG S2
RV3	CTGTTCTTTCCTGTTATCGC	See FIG S2

Table S15: obtained relative growth speed of tested hybrids, their respective parental strains and two commercial lager yeasts at different temperatures

	41°C	37°C	30°C	25°C	16°C	10°C	8°C	4°C
H1	0.0	207.0	234.5	217.0	372.5	219.5	193.5	271.0
H2	0.0	191.5	219.0	223.5	434.5	226.0	234.5	337.0
H3	0.5	191.0	223.5	227.5	410.5	211.0	194.0	138.5
H6	76.0	144.0	204.0	200.0	390.5	215.0	219.5	288.5
H4	0.0	177.0	221.0	207.0	371.5	185.5	198.5	271.5
H5	0.0	194.5	210.0	196.5	309.5	177.5	192.0	268.0
H7	0.0	215.0	222.5	208.5	396.0	203.0	205.0	284.0
H8	0.0	214.0	231.5	210.0	347.0	186.5	198.0	284.5
H9	15.5	188.0	225.0	213.0	429.5	208.0	197.0	0.0
H11	11.5	181.0	252.5	223.5	453.0	228.5	221.5	129.0
H10	0.0	177.0	223.0	224.5	446.5	226.5	223.5	298.5
H18	0.0	217.0	220.5	188.5	366.5	175.0	145.5	227.0
H19	1.5	216.0	212.5	199.0	336.0	139.0	115.5	128.5
H20	0.5	203.0	186.5	179.0	280.5	112.0	90.5	104.5
H23	0.5	231.0	232.0	216.5	403.5	205.0	148.5	136.0
H22	0.0	205.0	198.5	146.0	286.5	94.5	78.0	117.5
H21	0.0	239.5	210.0	184.0	381.0	147.5	113.0	97.0
H26	30.5	251.0	220.5	219.0	437.0	215.5	180.0	190.0
H25	0.5	190.0	194.5	198.0	379.5	184.5	160.5	142.0
H24	2.5	253.0	225.0	195.5	331.0	144.5	112.5	75.0
H27	16.5	227.0	215.5	184.0	387.0	157.0	124.5	130.5
H28	2.0	185.5	181.5	144.5	308.0	75.0	57.5	52.0
H29	1.5	258.0	228.0	213.0	358.5	174.0	154.0	116.5
H31	56.0	232.5	216.5	201.0	393.5	186.5	170.5	130.5
H30	0.0	190.0	187.5	187.5	399.5	180.0	165.0	101.5
H12	12.0	254.0	226.0	213.5	373.5	189.5	180.5	205.0
H14	18.5	241.0	204.0	203.5	329.0	154.0	139.0	148.0
H13	0.0	176.0	188.0	194.0	320.0	122.5	111.0	109.0
H15	0.0	175.0	183.5	122.0	211.5	54.0	35.5	43.0
H16	0.5	213.0	208.0	215.0	407.5	172.5	155.5	162.0
H17	0.0	211.0	224.0	225.5	443.0	183.0	172.0	177.5
Y134	275.0	250.5	214.5	190.0	290.0	119.0	93.5	9.0
Y245	246.0	281.5	211.5	209.0	356.0	118.5	99.0	0.0
Y377	382.5	241.0	195.5	230.0	325.0	163.0	0.0	0.0
Y397	281.0	270.5	213.0	186.0	296.0	83.0	59.0	10.5
Y470	716.0	276.5	250.5	229.5	382.0	143.5	135.5	74.5
GSY132	4.0	0.0	84.5	110.0	203.0	85.5	76.5	37.0
GSY501	0.0	0.0	5.5	155.5	408.5	243.0	233.0	564.0
Y184	873.5	253.5	251.0	229.5	455.0	169.0	134.5	68.5
Y565	0.0	0.0	204.5	217.5	337.5	215.0	222.5	290.0
Y567	0.0	0.0	193.5	204.0	348.5	199.0	194.5	272.5

7

List of publications

7.1 First author publications

Mertens, S., Steensels, J., Saels, V., De Rouck, G., Aerts, G., & Verstrepen, K. J. (2015). A large set of newly created interspecific yeast hybrids increases aromatic diversity in lager beers. *Applied and environmental microbiology*, AEM-02464.

Mertens, S., Steensels, J., Gallone, B., Souffriau, B., Malcorps, P., & Verstrepen, K. J. (2017). Rapid screening method for phenolic off-flavor (POF) production in yeast. *Journal of the American Society of Brewing Chemists*, *75*(4), 318-323.

Gallone, B*., **Mertens, S.***, Gordon, J. L., Maere, S., Verstrepen, K. J., & Steensels, J. (2018). Origins, evolution, domestication and diversity of Saccharomyces beer yeasts. *Current opinion in biotechnology*, *49*, 148-155.

Mertens, S., Gallone, B., Steensels, J., Herrera, B., Cortebeek, J., Nolmans, R., Saels, V., Vyas, V.K. and Verstrepen, K.J. (2018). Reducing phenolic off-flavors through CRISPR-based gene editing of the *FDC1* gene in *Saccharomyces cerevisiae* x *Saccharomyces eubayanus* hybrid lager beer yeasts (PLOSone, accepted manuscript)

7.2 Other publications

Snoek, T., Nicolino, M. P., Van den Bremt, S., **Mertens, S**., Saels, V., Verplaetse, A., ... & Verstrepen, K. J. (2015). Large-scale robot-assisted genome shuffling yields industrial Saccharomyces cerevisiae yeasts with increased ethanol tolerance. *Biotechnology for biofuels*, *8*(1), 32.

Vervoort, Y., Herrera-Malaver, B., **Mertens, S.**, Guadalupe Medina, V., Duitama, J., Michiels, L., ... & Verstrepen, K. J. (2016). Characterization of the recombinant Brettanomyces anomalus β-glucosidase and its potential for bioflavouring. *Journal of applied microbiology*, *121*(3), 721-733.

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