Optimizing the brewing yeast Saccharomyces pastorianus by genetic engineering

Bennis, N.X.

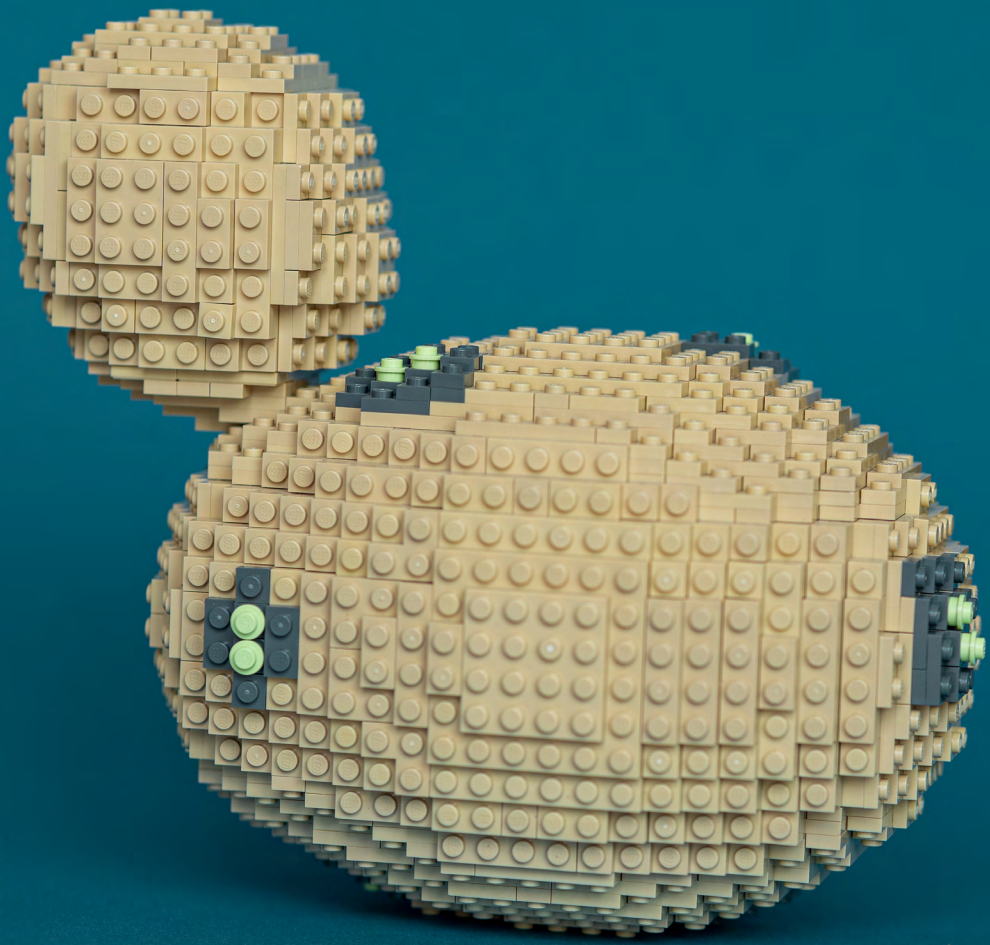
DOI
10.4233/uuid:ed4feab5-8b21-429c-a7fd-533f2354210e

Publication date
2024

Document Version
Final published version

Citation (APA)
Bennis, N. X. (2024). *Optimizing the brewing yeast Saccharomyces pastorianus by genetic engineering*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:ed4feab5-8b21-429c-a7fd-533f2354210e

Optimizing the brewing yeast *Saccharomyces pastorianus* by genetic engineering



Nicole X. Bennis

Optimizing the brewing yeast *Saccharomyces pastorianus* by genetic engineering

Dissertation

For the purpose of obtaining the degree of doctor
at Delft University of Technology
by the authority of the Rector Magnificus prof. dr. ir. T.H.J.J. van der Hagen,
chair of the Board for Doctorates,
to be defended publicly on
Friday 22 November 2024 at 12:30 o'clock.

by

Nicole Xanthe BENNIS

Master of Science in Life Science and Technology,
Delft University of Technology, The Netherlands
Born in Zoetermeer, The Netherlands

This dissertation has been approved by the promotors.

Composition of the doctoral committee:

Rector Magnificus	chairperson
Prof. dr. ir. J.M.G. Daran	TU Delft, promotor
Prof. dr. J.T. Pronk	TU Delft, promotor

Independent members:

Dr. V. Boer	HEINEKEN Supply Chain B.V.
Prof. dr. P. Branduardi	University of Milano-Bicocca, Italy
Prof. dr. ir. M. Ottens	TU Delft
Dr. C.E. Paul	TU Delft
Prof. dr. J.H. de Winde	Leiden Univeristy, The Netherlands
Prof. dr. ir. P.A.S. Daran-Lapujade	TU Delft, reserve member

The research presented in this thesis was performed at the Industrial Microbiology Section, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, The Netherlands.

This work was performed within the Top consortia for Knowledge and Innovation (TKIs) AgriFood which was granted a PPP allowance from the Ministry of Economic Affairs and Climate Policy (Project Habitats #PPPS1701).

Cover:	Maik Helgers & Nicole X. Bennis
Layout:	Nicole X. Bennis
Printed by:	Ridderprint, The Netherlands
ISBN:	978-94-6366-960-3

An electronic version of this dissertation is available at http://repository.tudelft.nl/

Table of Contents

Summary

The hybrid yeast *Saccharomyces pastorianus* is the microbial 'workhorse' responsible for production of lager beer. With an annual production of 1.9 billion hectolitres, this type of beer is the most popular alcoholic beverage worldwide. *S. pastorianus* ferments sugars present in wort to ethanol and a variety of flavour molecules that contribute to the taste and perception of the beverage. This yeast has emerged from a natural hybridization event in a human-caused environment, which probably occurred in late medieval times, between the mesophilic ale yeast *Saccharomyces cerevisiae* and the cryotolerant yeast *Saccharomyces eubayanus*. As a consequence, *S. pastorianus* strains harbour chromosomes from both these parental species. In addition, prolonged domestication in lager brewing processes has led to many additional changes in *S. pastorianus*: individual chromosomes underwent extensive recombination and occur in different copy numbers that, moreover, vary between strains. The advent of whole genome sequencing technologies has contributed to insights into the complex genomic 'blueprints' of *S. pastorianus* strains.

The allo-aneupolyploid genomes of currently used brewing yeast strains were shaped in centuries of evolution, with industrial brewing conditions providing selective pressure. Today, targeted genetic engineering enables researchers to alter or add traits beyond what is achievable by solely applying selective pressure. The rapid development of CRISPR-Cas-based tools for genome editing have, particularly in baker's yeast *S. cerevisiae* and to a lesser extent in non-conventional yeast species, greatly accelerated strain development. However, applying these tools in heterozygous allo-aneupolyploid contexts such as those in *S. pastorianus* poses substantial technological challenges.

The aim of the research described in this thesis was to develop novel genome editing strategies for yeasts, improving the genetic accessibility of hybrid yeasts and subsequently applying these methodologies to generate novel lager brewing strains with enhanced brewing characteristics.

Modern genome editing approaches, including CRISPR-Cas, typically exploit cellular pathways responsible for repair of DNA damage. Two main mechanisms participate in the repair of double-strand DNA breaks (DSB): non-homologous end joining (NHEJ) and homology-directed repair (HDR). The error-prone NHEJ system directly ligates the strand ends, with accompanying nucleotide insertions or deletions. In contrast, HDR repairs DSBs by using the genetic information from a DNA donor template. When the CRISPR-Cas endonuclease is targeted to a DNA sequence of interest by a specifically designed CRISPR RNA, a DSB is introduced, which activates the HDR machinery to repair the break by recombination with a naturally available DNA template or

a template provided by the researcher. Recent intensive research had led to the discovery of a variety of Cas endonucleases, among which the single effector proteins Cas9 and Cas12a, belonging to Class 2. To date, the most studied endonucleases are type-II *Streptococcus pyogenes* Cas9 (*Spy*Cas9) and type-V-A *Francisella novicida* Cas12a (*Fn*Cas12a) (previously known as (Cpf1).

Several CRISPR-Cas methods have been validated for fast and highly efficient single gene editing events in *S. cerevisiae*. Moreover, diverse multiplex genome editing approaches have been developed for both Cas9 and Cas12a endonucleases and their associated guide RNAs (gRNAs), which target these endonucleases to specific genomic locations. Typically, the gRNAs used to guide a Cas endonuclease to the editing site are expressed from plasmid-borne expression cassettes under control of native RNA polymerases II or III. However, the gRNA-expression plasmids are often suboptimal and their construction requires time-consuming cloning steps. In **Chapter 2**, the potential of expressing gRNA from linear DNA fragments using T7 RNA polymerase, both for single and multiplex editing, was investigated in *S. cerevisiae*. Transforming short, linear DNA fragments encoding gRNAs in yeast strains expressing a T7 RNA polymerase was shown to be adequate to direct the *Fn*Cas12a endonuclease to the site of action, resulting in highly efficient single and multiplexed gene editing. By introducing a T7 RNA polymerase and gDNA oligonucleotide, this approach decouples gRNA production from the host polymerase and plasmid templates, thereby completely eliminating these obstacles. The DNA fragments used in this new approach can be custom-ordered, which makes it highly suitable for automated, high-throughput yeast strain construction.

The deeply investigated and widely applied hallmark endonuclease *Spy*Cas9 is associated with a complex patent landscape and its targeting requirements, such as the requirement for a GC-rich PAM sequence and complex structural elements required for gRNAs, can restrict its applicability in some contexts. **Chapter 3** investigates application of a recently discovered Cas12a endonuclease for genome editing in *S. cerevisiae*, originating from *Eubacterium rectale*. Cas12 possesses attractive fundamental characteristics and its application is not coupled to reach-through royalty claims. Methods to improve the efficiency of *Eubacterium rectale* Cas12a (*Er*Cas12a) editing were investigated by adjusting the length of the direct repeats and spacers as well as by testing different crRNA expression systems. The optimal design parameters for genome editing were defined as a 21 nucleotide spacer flanked by 19 nucleotide direct repeats expressed from either RNA polymerase II or III promoters. Applying these guidelines to single editing events resulted in near 100% editing efficiencies. Moreover, the ability of *Er*Cas12a to process a multi-spacer crRNA array using its innate RNA processing capability allowed for the simultaneous editing of multiple chromosomal locations.

Both di- and polyploid heterozygous and hybrid yeast strains experience unpredictable loss of heterozygosity when introducing targeted DSBs with CRISPR-Cas in heterozygous chromosomes. In such scenarios, the intact homolog or homoeologous chromosome is often preferred as template for homology-directed repair, thus preventing the targeted introduction of the desired repair construct. This loss of heterozygosity undermines targeted genetic editing and drastically reduces genome editing efficiency in these yeasts. **Chapter 4** outlines how the extraordinarily complex hybrid genome of the lager yeast *S. pastorianus* can be edited with high efficiency by targeting Cas9 to carefully selected genomic 'landing sites'. The uniqueness of the chimeric *Se*Sc*CHRIII chromosome in the applied strain CBS 1483 was crucial for it to serve a such as safe platform for CRISPR-Cas9 editing. Integration sites on this chromosome were selected and evaluated based on i) absence of loss of heterozygosity upon CRISPR-editing, ii) efficiency of the gRNA, and iii) absence of effect on strain physiology. Successful examples of highly efficient single and double gene integration illustrate that genome editing can be applied in hybrid yeasts, thereby opening up new avenues for lager yeast strain development. Genome-wide analysis of different *S. pastorianus* strains showed that a chimeric *Se*Sc*CHRIII chromosome was nearly always present as sole CHRIII or in combination with either a *Se*CHRIII or *Sc*CHRIII version. This observation indicates that our results should be applicable to other *S. pastorianus* strains and interspecific hybrids and, thereby, can contribute to a rapidly intensifying field of research on improving and expanding performance of brewing yeasts by introduction of expression cassettes for non-native genes or gene versions. An example of such an application was investigated in detail in **Chapter 5**.

In **Chapter 5**, the landing sites on *Se*Sc*CHRII were used for metabolic engineering of *S. pastorianus* to enhance the biosynthesis of ethyl esters (ethyl hexanoate, ethyl octanoate and ethyl decanoate), which, even in trace amounts, are crucial flavour compounds in lager beer. Ethyl esters, known for imparting fruity and floral taste notes to beer, are synthesized from cytotoxic medium-chain acyl-CoA intermediates released by the fatty acid synthase complex during the fatty acid biosynthesis. Increasing the supply of medium-chain acyl-CoA precursors was identified as the prime target for increasing the ethyl ester levels. Through Cas9-based genetic engineering, specific mutations were introduced in domains of the fatty acid synthase complex encoded by *FAS1* and *FAS2* genes. The resulting variants were then overexpressed individually or in combination by integration of expression cassettes at the genomic 'landing sites' identified in **Chapter 4**. Overexpression of *ScFAS1*^{I306A} and *ScFAS2*^{G1250S} significantly improved ethyl hexanoate production while *ScFAS1*^{R1834K} boosted ethyl octanoate production. Combined overexpression of *FAS1* and *FAS2* genes carrying these specific mutations also increased final ethyl ester concentrations in cultures grown on full malt wort. Additional overexpression of the esterase gene *ScEEB1* increased the conversion of the increased pools of medium-chain fatty acids into the corresponding ethyl esters. Brewing tests with these genetically engineered *S. pastorianus* strains

confirmed significant alterations in the flavour profiles of the produced beers. This chapter highlights the potential of metabolic and genetic engineering in *S. pastorianus* for producing alternative flavour palettes in beer.

Samenvatting

De hybride gist *Saccharomyces pastorianus* is het microbiële 'werkpaard' dat verantwoordelijk is voor de productie van pilsbier. Met een jaarlijkse productie van 1,9 miljard hectoliter is dit type bier wereldwijd de populairste alcoholische drank. *S. pastorianus* fermenteert suikers die in wort aanwezig zijn tot ethanol en een verscheidenheid aan smaakmoleculen die bijdragen aan de smaak en perceptie van de drank. Deze gist is voortgekomen uit een natuurlijke hybridisatiegebeurtenis tussen de mesofiele biergist *Saccharomyces cerevisiae* en de cryotolerante gist *Saccharomyces eubayanus*. Deze hybridisatie vond hoogstwaarschijnlijk plaats in de late middeleeuwen in een door de mens veroorzaakte omgeving. Als gevolg van deze genetische oorsprong herbergen de *S. pastorianus*-stammen chromosomen van beide oudersoorten. Bovendien heeft langdurige domesticatie in brouwprocessen geleid tot veel extra veranderingen in *S. pastorianus*: individuele chromosomen ondergingen uitgebreide recombinatie en komen voor in verschillende kopie-aantallen die bovendien variëren tussen stammen. De komst van technieken waarmee de DNA-volgorde van complete genomen kan worden bepaald heeft in belangrijke mate bijgedragen aan het verkrijgen van inzicht in de complexe genetische 'blauwdrukken' van *S. pastorianus*-stammen.

De allo-aneupolyploïde genomen van de momenteel gebruikte brouwgiststammen zijn het resultaat van eeuwen van evolutie waarvoor industriële brouwomstandigheden de selectiedruk leverden. Tegenwoordig stelt gerichte genetische manipulatie onderzoekers in staat om eigenschappen te veranderen of toe te voegen die verder gaan dan wat haalbaar is door alleen selectieve druk uit te oefenen. De snelle ontwikkeling van op CRISPR-Cas gebaseerde hulpmiddelen voor genoombewerking ('genome editing', ofwel het herschrijven van delen van het genoom) heeft, met name in bakkersgist *S. cerevisiae* en in mindere mate in niet-conventionele gistsoorten, de ontwikkeling van stammen sterk versneld. Het toepassen van deze hulpmiddelen in complexe heterozygote en allo-aneupolyploïde contexten zoals die in *S. pastorianus*, brengt echter nog steeds aanzienlijke technologische uitdagingen met zich mee.

Het doel van het onderzoek dat in dit proefschrift wordt beschreven, was het ontwikkelen van nieuwe genoombewerkingsstrategieën voor gisten, het verbeteren van de genetische toegankelijkheid van hybride gisten en vervolgens het toepassen van deze methodologieën om nieuwe pilsbrouwstammen te genereren met verbeterde brouweigenschappen.

Moderne benaderingen voor genoombewerking, waaronder CRISPR-Cas, maken meestal gebruik van cellulaire routes die verantwoordelijk zijn voor het herstel van DNA-schade. Twee belangrijke mechanismen nemen deel aan het herstel

van dubbelstrengs DNA-breuken (DSB): 'non-homologous end joining' (NHEJ) en 'homology directed repair' (HDR). Het foutgevoelige NHEJ-systeem ligeert de uiteinden van de strengen rechtstreeks, waarbij vaak nucleotide-inserties of -deleties optreden. HDR daarentegen repareert DSB's door de genetische informatie van een DNA-donorsjabloon te gebruiken. Wanneer de CRISPR-Cas-endonuclease wordt gericht op een bepaalde DNA-sequentie door een specifiek ontworpen CRISPR-RNA, wordt een DSB geïntroduceerd. De DNA-breuk activeert de HDR mechanisme om de breuk te repareren door middel van recombinatie met een natuurlijk beschikbaar of door de onderzoeker aangeleverd DNA-sjabloon. Recent intensief onderzoek heeft geleid tot de ontdekking van een verscheidenheid aan Cas-endonucleases, waaronder de eiwitten Cas9 en Cas12a die tot klasse 2 behoren. Tot op heden zijn de meest bestudeerde endonucleases type-II *Streptococcus pyogenes* Cas9 (*Spy*Cas9) en type-V-A *Francisella novicida* Cas12a (*Fn*Cas12a) (voorheen bekend als Cpf1).

Verschillende CRISPR-Cas-methoden zijn gevalideerd voor snelle en efficiënte van enkelvoudige genetische modificaties in *S. cerevisiae*. Bovendien zijn er diverse multiplex genoombewerkingsbenaderingen (ingrepen waarbij tegelijkertijd verschillende genetische modificaties worden aangebracht) ontwikkeld voor zowel Cas9- als Cas12a-endonucleases en hun bijbehorende gids-RNA's (gRNA's). Deze gRNA's zorgen ervoor dat de endonucleases worden gericht op specifieke locaties in het genoom. Doorgaans worden de gRNA's die worden gebruikt om een Cas-endonuclease naar de bewerkingsplaats te leiden, tot expressie gebracht vanaf expressiecassettes die op plasmiden worden aangeboden en waarbij de expressie wordt gecontroleerd door natieve RNA-polymerases II of III. De plasmiden met gRNA-expressie zijn echter vaak suboptimaal en hun constructie vereist tijdrovende kloneringsstappen. In **hoofdstuk 2** werd daarom een alternatieve methode onderzocht. Hierbij werd het gRNA in *S. cerevisiae* afgelezen van lineaire DNA-fragmenten met behulp van T7 RNA-polymerase, zowel voor enkelvoudige als multiplex genoombewerking. Het transformeren van korte, lineaire DNA-fragmenten die coderen voor gRNA's in giststammen die een T7 RNA-polymerase tot expressie brengen, bleek voldoende te zijn om de *Fn*Cas12a-endonuclease naar de plaats van actie te leiden, wat resulteerde in zeer efficiënte enkelvoudige en multiplex genbewerking. Door een T7 RNA-polymerase en gDNA-oligonucleotide te introduceren, omzeilt deze methode volledig de noodzaak voor het gebruik van een gistpolymerase en voor plasmideconstructie. De DNA-fragmenten die in deze nieuwe aanpak worden gebruikt, kunnen op maat worden besteld, waardoor deze methode zeer geschikt is voor geautomatiseerde 'high-throughput' constructie van giststammen.

De grondig onderzochte en op grote schaal toegepaste endonuclease *Spy*Cas9 is geassocieerd met een complex octrooilandschap. Bovendien stelt dit nuclease specifieke eisen voor het correct sturen van het gRNA, zoals een GC-rijke PAM-sequentie en complexe structurele elementen in het gRNA. Deze eisen kunnen de

toepasbaarheid van *Spy*Cas9 in sommige contexten beperken. **Hoofdstuk 3** onderzoekt de toepassing van een recent ontdekte Cas12a-endonuclease voor genoombewerking in *S. cerevisiae*, afkomstig uit *Eubacterium rectale*. *Eubacterium rectale* Cas12a (*Er*Cas12) bezit aantrekkelijke fundamentele kenmerken en de toepassing ervan is niet gekoppeld aan 'reach-through' claims voor betaling van royalties. Methoden om de efficiëntie van *Er*Cas12a-bewerking te verbeteren werden onderzocht door de lengte van 'direct repeats' en spacers aan te passen en door verschillende gRNA-expressiesystemen te testen. De optimale ontwerpparameters voor genoombewerking werden gedefinieerd als een spacer met 21 nucleotiden geflankeerd door 'direct repeats' van 19 nucleotiden die tot expressie worden gebracht door RNA-polymerase II- of III-promoters. Het toepassen van deze richtlijnen op één genoomaanpassing resulteerde in bijna 100% bewerkingsefficiëntie. Bovendien maakte het vermogen van *Er*Cas12a om een multi-spacer crRNA-array te verwerken met behulp van zijn inherente RNA-verwerkingscapaciteit gelijktijdige modificaties op meerdere plekken in het genoom mogelijk.

Zowel di- als polyploïde heterozygote en hybride giststammen ondergaan een onvoorspelbaar verlies van heterozygotie bij het introduceren van gerichte dubbelstrengs-breuken met CRISPR-Cas in heterozygote chromosomen. In dergelijke scenario's blijkt vaak een intact homoloog of homeoloog chromosoom als sjabloon voor de DNA reparatie met HDR te worden gebruikt, waardoor de gerichte introductie van het door de onderzoeker ontworpen reparatieconstruct niet optreedt. Het resulterende verlies van heterozygotie ondermijnt gerichte genetische bewerking en vermindert de efficiëntie van het bewerken van het genoom in deze gisten drastisch. **Hoofdstuk 4** beschrijft hoe het buitengewoon complexe hybride genoom van de pilsbiergist *S. pastorianus* met hoge efficiëntie kan worden bewerkt door Cas9 te richten op zorgvuldig geselecteerde genomische 'landingsplaatsen'. Het unieke karakter van het chimeer *Se*Sc*CHRIII*-chromosoom in de hierbij gebruikte stam CBS 1483 was cruciaal om zo'n veilig platform te bieden voor CRISPR-Cas9-bewerking. Integratieplaatsen op dit chromosoom werden geselecteerd en geëvalueerd op basis van i) afwezigheid van verlies van heterozygotie bij CRISPR-bewerking, ii) efficiëntie van het gRNA, en iii) afwezigheid van effect op de fysiologie van de stam. Succesvolle voorbeelden van zeer efficiënte enkele en dubbele integratie van genen demonstreerden dat deze strategie voor genoombewerking kan worden toegepast in hybride gisten, waardoor nieuwe wegen worden geopend voor de ontwikkeling van pilsgiststammen. Genoombrede analyse van verschillende *S. pastorianus*-stammen toonde aan dat een chimeer *Se*Sc*CHRIII*-chromosoom bijna altijd aanwezig was, ofwel als enig CHRIII, ofwel in combinatie met een *Se*CHRIII- of *Sc*CHRIII-versie. Deze waarneming geeft aan dat onze resultaten toepasbaar zouden moeten zijn op andere *S. pastorianus*-stammen en hybriden en daardoor kunnen bijdragen aan een snelle intensivering van het onderzoek naar het verbeteren en uitbreiden van de prestaties van biergisten door de introductie van expressiecassettes voor (versies van) genen die van nature

niet in deze gisten voorkomen. Een voorbeeld van een dergelijke toepassing is in **hoofdstuk 5** uitvoerig onderzocht.

In **hoofdstuk 5** werden de "landingsplaatsen" op *Se*Sc*CHRIII gebruikt voor genetische modificatie van *S. pastorianus* met het doel om de biosynthese van ethylesters (ethylhexanoaat, ethyloctanoaat en ethyldecanoaat) te verbeteren. Deze verbindingen zijn zelfs in sporenhoeveelheden cruciale smaakstoffen in pilsbier. Ethylesters, die fruitige en bloemige smaaktonen geven in bier, worden gesynthetiseerd uit cytotoxische middellange-keten acyl-CoA-tussenproducten die vrijkomen tijdens de biosynthese van vetzuren door het vetzuursynthasecomplex. Het verhogen van het aanbod van middellangeketen-acyl-CoA-precursoren werd aangemerkt als een belangrijk doelwit voor het verhogen van de ethylesterniveaus. Door middel van genetische modificatie met Cas9 werden specifieke mutaties geïntroduceerd in domeinen van het vetzuursynthasecomplex dat wordt gecodeerd door de *FAS1*- en *FAS2*-genen. De resulterende varianten werden vervolgens afzonderlijk of in combinatie tot overexpressie gebracht door integratie van expressiecassettes op de in **hoofdstuk 4** geïdentificeerde 'landingsplaatsen'. Overexpressie van *ScFAS1*^{I306A} en *ScFAS2*^{G1250S} verbeterde de productie van ethylhexanoaat aanzienlijk, terwijl *ScFAS1*^{R1834K} de productie van ethyloctanoaat verhoogde. De gecombineerde overexpressie van *FAS1*- en *FAS2*-genen met deze specifieke mutaties verhoogde ook de uiteindelijke ethylesterconcentraties in culturen die op wort werden gekweekt. De combinatie van *FAS1*- en *FAS2*-overexpressie met overexpressie van het esterase-gen *ScEEB1* verhoogde de conversie van de verhoogd beschikbare middellange ketenvetzuren in de overeenkomstige ethylesters. Brouwproeven met deze genetisch gemanipuleerde *S. pastorianus*-stammen bevestigden significante veranderingen in de smaakprofielen van de geproduceerde bieren. Dit hoofdstuk belicht het potentieel van metabole en genetische manipulatie in *S. pastorianus* voor het produceren van alternatieve smaakpaletten in bier.

Chapter 1

Introduction

1

1.1. From traditional lagers to exploring the potential of alternative beers

Archaeological evidence traces the origins of brewing back to between 13000 and 4000 BCE (Michel *et al.*, 1992, Liu *et al.*, 2018). The Sumerians of Mesopotamia are among the first humans to have recorded their beer brewing activities (Hornsey, 2012). Ancient brewing processes were probably quite simple and based on spontaneous fermentation of grains in water. The Egyptian, Greek and Roman civilizations contributed to the technological development and spread of beer brewing. A further development of beer brewing occurred in medieval European monasteries. Monks not only brewed beer for their own consumption, but also shared their expertise with local communities. The use of hops as a flavouring and preservative agent, popularized in the 9th and 10th centuries, revolutionized the brewing process and contributed to development of the beer types we know today.

The emergence of the lager beer can be traced back to 16th-century Bavaria, where regulations were implemented to standardize the brewing process and improve product quality. The 'Reinheitsgebot' (or Purity Law, 1516) restricted the ingredients used in brewing to water, barley and hops and contributed to a more consistent product quality (Hornsey, 2003). Although not explicitly mentioned in the Reinheitsgebot, yeast played an essential role during fermentation. Despite instatement of the Reinheitsgebot, beer brewed in summer often exhibited a high degree of sourness. In 1533, an additional decree prohibited brewing from the Feast of Saint George (April 23th) to Michaelmas (September 29th). This decree effectively moved brewing activities to the colder season. Consequently, brewing yeasts were exposed to lower temperatures and, over time, bottom-fermenting yeasts (lager yeasts) became dominant. Bottom fermentation is done at lower temperature (5-10 °C), than top fermentations (18-25 °C) with ale yeasts. Due to the lower temperature, fermentation and carbon dioxide production in lager brewing processes are slower and yeast cells are able to form flocs that sediment to the bottom of the fermentation tank at the end of the fermentation process. The German word 'lagern' (to store) originated from the practice of storing winter-brewed beers during summer (Meussdoerffer, 2009).

The discoveries of Pasteur (Pasteur, 1858, Pasteur, 1876) and Hansen (Hansen, 1883) attributed alcoholic fermentation to the activity of yeast cells and led to the isolation of pure cultures of brewing yeasts. This development revolutionized the brewing process by improving consistency and quality of the produced beer. Technological advancements such as steam power and refrigeration further improved and intensified production and distribution of beer in the 19th and 20th centuries (Poelmans & Swinnen, 2011, Hutzler *et al.*, 2023). Large-scale breweries allowed for mass production and wider availability of beer to the growing urban populations. Today lager beer is by far the most popular beer type worldwide and, with a global consumption of 1.9 billion hectolitres in 2022, accounted for over 90% of total beer consumption (Kirin-Holdings,

2022). Except for a slight decline in 2020 due to the impact of the COVID-19 pandemic, the global lager beer market has experienced consistent growth and is predicted to reach over one trillion USD by 2030 (Fortune-Business-Insights, 2023).

In recent decades, the brewing industry has experienced significant transformations due to changing consumer preferences. A notable recent trend is the rise of craft brewing, which focuses on producing distinctive and innovative beers with an emphasis on quality and unique flavour profiles. By incorporating an array of additives, including fruits, spices, herbs, and various grains, non-conventional beer styles have been developed that cater for the diverse preferences of today's consumers. The market value for craft brewing is expected to double from 2020 to 2028 (Fortune-Business-Insights, 2023). Another significant trend, driven by more health- and safety-conscious consumers, is an increasing demand for low-alcohol and non-alcoholic beers. Brewers respond to this trend by developing beers with reduced alcohol content, while minimizing compromises on aroma and taste. Furthermore, the brewing industry actively seeks to reduce environmental impact of brewing by water conservation measures, implementation of more energy-efficient brewing processes and elimination of energy-intensive steps in production and transport (https://www.carlsberggroup.com/ and https://www.theheinekencompany.com/).

1.2. The importance of the *Saccharomyces* genus for industrial biotechnology and fundamental research

Among the nearly 2000 yeast genera identified, the *Saccharomyces* (Greek for 'sugar fungus') genus, belonging to the *Ascomycota* phylum, comprises a diverse group of species with impacts in various biological fields (Rainieri *et al.*, 2003) (Figure 1.1). The best known and most intensively investigated species in the *Saccharomyces* genus is *Saccharomyces cerevisiae*. Commonly known as baker's yeast, this yeast plays an indispensable role in human civilization (Legras *et al.*, 2007). First described in 1837 (Schwann, 1837, Donalies *et al.*, 2008), *S. cerevisiae* is a widely used industrial host for biotechnological processes due to its efficient metabolism for the conversion of sugars during alcoholic fermentation (Verstrepen *et al.*, 2006, Donalies *et al.*, 2008). This capability is not only crucial for the production of alcoholic beverages, but also for leavening bread, sustainable bioethanol production as alternative for petroleum-based fuels, and serving as host strain in various applications within the pharma-, agriculture-, and environmental biotechnology sectors.

S. cerevisiae is not only a versatile industrial microorganism but, as a eukaryotic model organism, also provides valuable insights into fundamental cellular processes in eukaryotes. In the latter role, *S. cerevisiae* has been at the forefront of molecular biology for five decades and played key roles in many cell biological and biotechnological breakthroughs, including discoveries in DNA replication, cell cycle regulation (Culotti &

Hartwell, 1971, Hartwell, 1971a, Hartwell, 1971b) (Nobel prize 2001, Leland H. Hartwell, Tim Hunt and Sir Paul M. Nurse), transcription (Lue & Kornberg, 1987) (Nobel prize 2006, Roger Kornberg) and telomere structure (Shampay *et al.*, 1984) (Nobel Prize 2009, Jack Szostak, Elizabeth Blackburn, and Carol Greider). The remarkably high genetic conservation of basic cellular processes in human cells and yeasts became apparent in 1987, when a human gene was shown to complement a mutation in yeast (Lee & Nurse, 1987). This observation opened the way to functionally analyse human genes and processes in the experimentally better accessible microorganism *S. cerevisiae* (Boonekamp *et al.*, 2022). *S. cerevisiae* boasts several firsts in fundamental and applied biology: the first eukaryote to have its genome fully sequenced (Goffeau *et al.*, 1996), the first genetically modified organism approved for food applications (Aldhous, 1990) and the first eukaryote carrying a fully synthetic chromosome (Annaluru *et al.*, 2014). Thorough study of this microorganism has led to an abundance of freely available datasets covering its transcriptome (Lashkari *et al.*, 1997, Cho *et al.*, 1998, Daran-Lapujade *et al.*, 2009), proteome (Zhu *et al.*, 2001) and metabolome (Jewison *et al.*, 2012) for a wide variety of genetic and environmental contexts. Moreover, a collection of *S. cerevisiae* deletion mutants (Winzeler *et al.*, 1999, Giaever *et al.*, 2002), genome-wide overexpression libraries (Jones *et al.*, 2008), and libraries of fluorescent-protein tagged yeast strains (Ghaemmaghami *et al.*, 2003, Huh *et al.*, 2003) are available (Cherry *et al.*, 2011).

Alongside *Saccharomyces cerevisiae*, the *Saccharomyces* genus includes the species *S. uvarum* (Pulvirenti *et al.*, 2000, Nguyen & Gaillardin, 2005), *S. paradoxus*, *S. kudriavzevii* (Sampaio & Gonçalves, 2008), *S. mikatae* (Naumov *et al.*, 2000), *S. arboricola* (Wang & Bai, 2008), *S. eubayanus* (Libkind *et al.*, 2011), the newest member of this genus, *S. jurei* (Dujon & Louis, 2017, Naseeb *et al.*, 2017), and interspecific hybrids designated *S. pastorianus* and *S. bayanus*, each with their own genotypic and phenotypic characteristics (Figure 1.1).

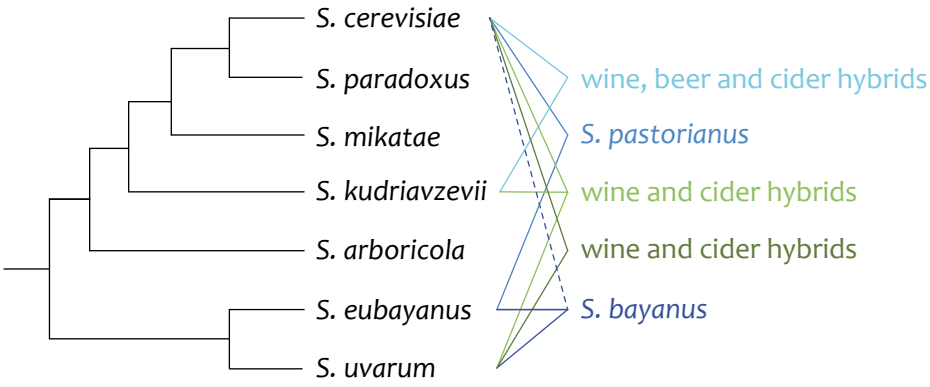


Figure 1.1. Phylogeny of the *Saccharomyces* genus based on genome sequencing data. Interspecific hybridizations resulting in *S. pastorianus* and *S. bayanus* species, as well as other hybrids found in wine, beer and cider fermentations are highlighted.

1.3. The emergence of lager brewing yeasts

S. cerevisiae is responsible for conversion of sugars into alcohol and flavours during the production of ale beers. Throughout the domestication of ale yeasts, variants were selected in which mutations conferred adaptations to changes in the brewing process. When brewing in the cold seasons was adopted and lager beer was developed, strong evolutionary pressures on the yeast drove a hybridization event between different *Saccharomyces* species. Hybridization is a natural mechanism that occurs upon mating between species (interspecific mating) and which leads to the combination of genomes of different species into a single cell. Thereby, hybridization allows the inheritance of phenotypic traits from both parental species but also can lead to hybrid vigour (heterosis), which encompasses the outperformance by the hybrid of both its parents (Gibson & Liti, 2015, Hebly *et al.*, 2015, Krogerus *et al.*, 2015). The interspecific hybrid yeast now referred to as *Saccharomyces pastorianus* is a hybrid of an *S. cerevisiae* ale-brewing yeast and the cold-tolerant species *S. eubayanus*. This hybrid proved more resilient to the fermentation conditions during lager brewing than either of its parental species (Nilsson-Tillgren *et al.*, 1981, Holmberg, 1982, Martini & Kurtzman, 1985, Martini & Martini, 1987, Tamai *et al.*, 1998, Yamagishi & Ogata, 1999). Other hybrid yeast species, such as hybrids of *S. cerevisiae* and either *S. kudriavzevii*, *S. uvarum* or the hybrid *S. bayanus* and even triple hybrids of *S. uvarum*, *S. cerevisiae* and *S. kudriavzevii*, have been identified mostly in wine, beer and cider fermentation contexts (Masneuf *et al.*, 1998, Naumov *et al.*, 2000, Naumova *et al.*, 2005, Querol & Bond, 2009, Libkind *et al.*, 2011) (Figure 1.1).

As a result of the domestication and identification of favourable fermentation traits, distinct pure culture lineages of the lager-brewing yeast were isolated, characterized and classified (Gallone *et al.*, 2016). Taxonomically, these hybrid, bottom-fermenting yeasts were classified as *S. carlsbergensis* (Unterhefe Nr. I), *S. pastorianus* and *S. monacensis* (Unterhefe Nr. II) (Hansen, 1896, Meussdoerffer, 2009, Monerawela & Bond, 2017). These species were initially treated as separate entities due to their distinct fermentation characteristics and physiological differences (Kyselová *et al.*, 2023), but in the late 1980s, it was discovered that all bottom-fermenting yeasts were similar interspecific hybrid yeasts and they were collectively reclassified as *S. pastorianus* (Nilsson-Tillgren *et al.*, 1981, Martini & Martini, 1987, Martini & Martini, 1998, Rainieri *et al.*, 2006, Bond, 2009, Wendland, 2014, Gibson & Liti, 2015, Kyselová *et al.*, 2023). Only recently, the genome of *S. pastorianus* was shown to encompass sequences of *S. cerevisiae* and of an initially unknown *Saccharomyces* species (Holmberg, 1982, Casaregola *et al.*, 2001, Naumova *et al.*, 2005, Rainieri *et al.*, 2006, Caesar *et al.*, 2007, Smart, 2007, Dunn & Sherlock, 2008, Bond, 2009). For a long time, it was believed that this missing parental species was represented by a variant of the hybrid yeast *Saccharomyces bayanus* (Martini & Martini, 1987, Tamai *et al.*, 1998, Yamagishi & Ogata, 1999, Casaregola *et al.*, 2001, Rainieri *et al.*, 2006, Nakao *et al.*, 2009), a more

cold-tolerant yeast species (Sato *et al.*, 2002). The first whole genome sequence of the lager brewing yeast *S. pastorianus* was obtained from Weihenstephan 34/70, commonly used in lager beer brewing (Nakao *et al.*, 2009). The *S. cerevisiae* content of the genome assembly had an average of 99.2% identity with the genome of the *S. cerevisiae* laboratory strain S288C, but its *S. bayanus*-like content showed a relatively low similarly with only 92.7% average identity to the open reading frames of the *S. bayanus* CBS 7001 reference strain (the only *S. bayanus* genome sequence available at the time). This indicated that *S. bayanus* was not the direct ancestor of *S. pastorianus* and not involved in the hybridization event (Monerawela & Bond, 2017).

In 2011, the first natural isolate of *S. eubayanus*, was found on fruiting bodies of *Cyttaria hariotii* growing on *Nothofagus spp.* trees in the Patagonian Andes in Argentina (Libkind *et al.*, 2011, Sampaio, 2018). Sequencing of its genome instantaneously untangled the mystery around the missing parent of the lager brewing yeast *S. pastorianus*, as it showed a 99.5% similarity to the non-*cerevisiae* subgenome (Libkind *et al.*, 2011, Sampaio, 2022). With the discovery of *S. eubayanus*, *S. bayanus* is known to result from the hybridization between *S. uvarum* and *S. eubayanus*, with introgressions of *S. cerevisiae* (Libkind *et al.*, 2011). Since its discovery, *S. eubayanus* has been found at more locations in Patagonia (Rodríguez *et al.*, 2014, Nespolo *et al.*, 2020), the Tibetan Himalayas, Sichuan and West China (Bing *et al.*, 2014), New Zealand (Gayevskiy & Goddard, 2016), North America (Peris *et al.*, 2014, Langdon *et al.*, 2020) and Ireland (Bergin *et al.*, 2022). Next-generation sequencing advances were essential to reconstruct the genome of *S. eubayanus* (Libkind *et al.*, 2011, Baker *et al.*, 2015, Hebly *et al.*, 2015, Gayevskiy & Goddard, 2016, Brouwers *et al.*, 2019, Langdon *et al.*, 2020, Bergin *et al.*, 2022). Phylogenetic analysis of the isolated *S. eubayanus* strains revealed that the *S. eubayanus* subgenome of *S. pastorianus* is more closely related to strains belonging isolated from the Himalayans (Bing *et al.*, 2014, Gibson & Liti, 2015, Peris *et al.*, 2016, Langdon *et al.*, 2020, Bergin *et al.*, 2022). However, no known *S. eubayanus* strain isolated until today seems to be the direct ancestor of lager-brewing yeasts while, based on sequence similarity, ale strains are the most likely contributor to their *S. cerevisiae* subgenome (Gallone *et al.*, 2016, Gonçalves *et al.*, 2016). Genome-wide analysis of *S. cerevisiae* isolates clearly separates into distinctive lineages by geographical locations or by source type. The beer-related *S. cerevisiae* strains cluster as the likely ancestor of lager yeasts (Gallone *et al.*, 2016, Monerawela & Bond, 2017).

1.4. The complex genome architecture of *S. pastorianus*

After the first *S. pastorianus* genome assembly (Nakao *et al.*, 2009), genomes of *S. pastorianus* isolated from the Carlsberg brewery (*S. carlsbergenesis* Unterhefe Nr. 1, now in the Centraal Bureau voor Schimmelcultures as CBS 1513) (Walther *et al.*, 2014) and *S. pastorianus* CBS 1483 isolated from the Heineken brewery (van den Broek *et al.*, 2015, Salazar *et al.*, 2019) were, amongst others (Hewitt *et al.*, 2014, De León-Medina *et al.*,

2016), sequenced using next-generation sequencing techniques. Among *S. pastorianus* strains, significant variability is observed between their brewing characteristics. This variability is particularly evident in their ability to ferment maltotriose and in their growth kinetics at low temperatures. Consequently, *S. pastorianus* strains were classified into two primary groups: Saaz (Group I) and Frohberg (Group II) (Liti *et al.*, 2005, Dunn & Sherlock, 2008). These groups correlate with geographical origin of *S. pastorianus* strains. Group I strains (sometimes referred to as *S. carlsbergensis* (Wendland, 2014)) were used in the Carlsberg brewery (Denmark) and in Saaz (Czech Republic) beer types, while Group II contains strains from the Netherlands (Heineken, Oranjeboom and other breweries), non-Carlsberg Danish breweries and two North American breweries (Dunn & Sherlock, 2008). Both lineages harbour interspecific allopolyploid hybrids derived from hybridization of *S. cerevisiae* and *S. eubayanus* (Dunn & Sherlock, 2008, Gibson *et al.*, 2013, Walther *et al.*, 2014, van den Broek *et al.*, 2015, Salazar *et al.*, 2019). Group I and Group II strains both harbour a diploid genome originating from *S. eubayanus*. A contrast emerges in their *S. cerevisiae* component, which is incomplete in Group I strains (for example, *Sc*CHRVI, *Sc*CHRXI and *Sc*CHRXII are often missing), while Group II strains contain a diploid or even larger *S. cerevisiae* genome complement (Liti *et al.*, 2005, Dunn & Sherlock, 2008, Wendland, 2014, van den Broek *et al.*, 2015, Okuno *et al.*, 2016, Gorter de Vries *et al.*, 2019). This genetic disparity corresponds with phenotypic attributes observed within these groups. Group I strains, whose genomes show a preponderance of *S. eubayanus* content, display higher cold-temperature adaptability, but a poorer fermentation performance than Group II strains, which is probably due to compromised maltotriose utilization (Gibson *et al.*, 2013). Conversely, the genetic makeup of Group II strains accentuates qualities inherent to *S. cerevisiae,* including more efficient maltotriose utilization, albeit often at the expense of low-temperature performance (Gibson *et al.*, 2013, Gallone *et al.*, 2016).

Chromosome copy numbers can directly affect phenotype, since increased gene copy number affects expression levels and, in addition, can confer mutational flexibility (De Barros Lopes *et al.*, 2002, Liti *et al.*, 2005, Krogerus *et al.*, 2016, Gorter de Vries *et al.*, 2017, De La Cerda Garcia-Caro *et al.*, 2022). Possessing additional gene copies expands the potential for functional enhancements and gene neofunctionalization, allowing for the emergence of novel functions. In *S. pastorianus*, chimeric chromosomes (e.g. *Se*Sc*CHRIII, *Sc*Se*CHRVII, *Se*Sc*CHRVII, *Sc*Se*CHRXIII and *Se*Sc*CHRXIII) were formed as a result of the recombination of homologous chromosomes inherited from their two different parents (Bond *et al.*, 2004, Dunn & Sherlock, 2008, Bond, 2009, Nakao *et al.*, 2009, Salazar *et al.*, 2019). In these chimeric chromosomes, recombination sites are often located in open reading frames, leading to the emergence of new unique chimeric genes (e.g. *ZUO1*, *KEM1*/*XRN1*, *HSP82*, *MAT* and *MALT413*) (Hewitt *et al.*, 2014, Walther *et al.*, 2014, Okuno *et al.*, 2016, Brouwers *et al.*, 2019). Chimeric genes can confer new or improved functions or can be regulated differently compared to the original genes and might play important roles in hybrid vigour (Hewitt *et al.*, 2014, Gorter de Vries *et al.*,

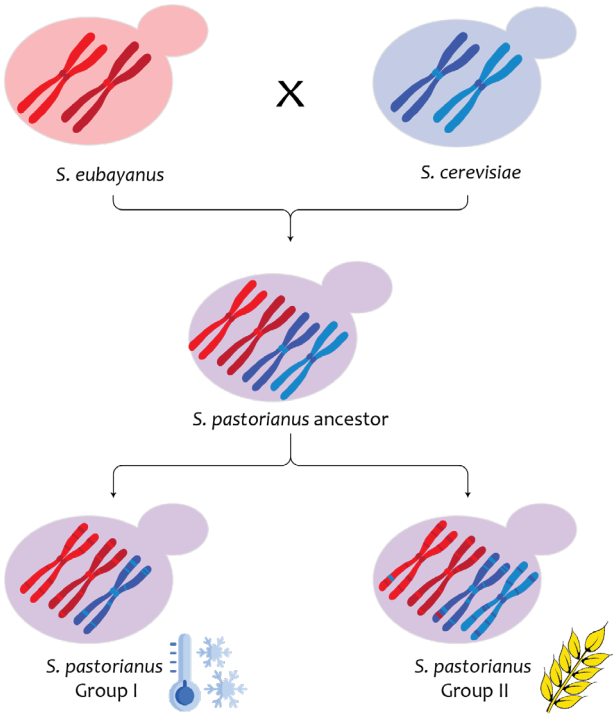


Figure 1.2. Hybridization of the cryotolerant *S. eubayanus* cell and an *S. cerevisiae* cell resulted in a common *S. pastorianus* ancestor from which two genotypically and phenotypically distinct groups evolved. Group I and Group II strains of *S. pastorianus* exhibit a diploid genome originating from *S. eubayanus*. Group II strains retained both *S. cerevisiae* derived chromosomes, Group I strains have a smaller and often incomplete *S. cerevisiae* subgenome. Group I strains are better adapted to low-temperatures, but exhibit poorer fermentation performance, while Group II strains have better maltotriose-fermentation, typically at the expense of low-temperature resilience.

2019). A consequence of harbouring a hybrid genome is that its genetic redundancy causes genetic instability (Smart, 2007, Querol & Bond, 2009, Kumaran *et al.*, 2013). Particularly, the repetitive (sub)telomeric regions of chromosomes, which often harbour genes involved in industrially relevant traits such as flocculation and sugar uptake (e.g. *FLO* genes and *MAL* loci), are relatively unstable and provide room for genetic divergence (Monerawela *et al.*, 2015, Monerawela & Bond, 2017).

How hybridization and/or subsequent changes in hybrid genomes resulted in the two distinct groups has been subject of intensive debate in literature (Querol & Bond, 2009, Monerawela & Bond, 2017, Gallone *et al.*, 2018, Monerawela & Bond, 2018, Salazar *et al.*, 2019) (Figure 1.2). One hypothesis suggests that the two distinct groups emerged from two independent single hybridization events. According to this hypothesis, Group I strains emerged from mating of a haploid *S. cerevisiae* cell with a diploid *S. eubayanus* cell, while Group II strains emerged from mating of a haploid ale *S. cerevisiae* strain with a haploid *S. eubayanus* strain followed by whole genome duplication or hybridization

of two diploid parental strains (De Barros Lopes *et al.*, 2002, Rainieri *et al.*, 2006, Legras *et al.*, 2007, Dunn & Sherlock, 2008, Baker *et al.*, 2015). However, genome sequencing revealed identical recombination locations between *S. cerevisiae* and *S. eubayanus* chromosomes in Group I as well as Group II *S. pastorianus* strains for the genes *KEM1/XRN1*, *HSP82* and *MAT* (Hewitt *et al.*, 2014, Walther *et al.*, 2014, Okuno *et al.*, 2016, Monerawela & Bond, 2018, Salazar *et al.*, 2019). This observation of genetic markers pointed towards one common hybrid ancestor for both groups, after which the two groups diverged by changing chromosome copy numbers. The single hybridization event generating the *S. pastorianus* ancestor could have occurred between a haploid *S. cerevisiae* and diploid *S. eubayanus* similar to Group I strains, followed by a second hybridization event with a haploid *S. cerevisiae* to generate the Group II strains (Monerawela & Bond, 2017, Karabin *et al.*, 2018, Monerawela & Bond, 2018, Lin *et al.*, 2021). Differences within the *S. cerevisiae* subgenome led to the hypothesis that one of the parents in this second hybridization could have been a Stout-derived *S. cerevisiae* yeast strain (Monerawela & Bond, 2017, Monerawela & Bond, 2018). Nonetheless, the hypothesis remained that due to fragile sites or recombination hotspots, these identical chromosomal breakpoints could have emerged in two independent lineages (Hewitt *et al.*, 2014, Baker *et al.*, 2015, Monerawela *et al.*, 2015, Monerawela & Bond, 2017, Monerawela & Bond, 2018). Laboratory evolution of *S. cerevisiae* × *S. eubayanus* hybrids under lager brewing conditions however did not develop these genetic markers, which may be interpreted as support for the single-hybridization hypothesis (Gorter de Vries *et al.*, 2019). The recent increase in accessibility and affordability of whole-genome sequencing techniques has resulted in a boost in sequencing of *S. pastorianus* genomes, allowing for a large-scale comparative genome analysis between Group I and Group II strains. These studies showed that the DNA inherited from the *S. cerevisiae* is highly similar in all tested strains (Magalhães *et al.*, 2016, Okuno *et al.*, 2016, Gorter de Vries *et al.*, 2019, Salazar *et al.*, 2019) and, thereby, further supported the single-hybridization model.

1.5. Genetic targets for brewing yeast improvements

Currently used brewing yeasts are the results of centuries of evolution. Modifying the yeast genome in the laboratory allows for altering traits beyond what is achievable by solely applying selective pressure. Due to the rapid expansion of yeast genetic engineering tools in the recent decade, construction of genetically modified strains has become much faster as well as more accurate. This development paves the way for designing and constructing industrial brewing yeast strains with improved flavour profiles or other features related to product quality, consistency and product diversity (Saerens *et al.*, 2010, Dzialo *et al.*, 2017). In additional, genetic engineering targets for brewing strain development may be selected with the primary aim to increase the efficiency and sustainability of production processes.

Enhancing flavour profiles

Reducing or eliminating the off-flavours vicinal diketones, 4-vinyl guaiacol and hydrogen sulfide

The quality of the end product and, in particular, a well-balanced flavour profile, with a body and mouthfeel fitting to the targeted beer type along with the corresponding alcohol levels, is a primary target for process development and optimization. Reducing off-flavours and strengthening desired flavours is essential to achieve a good quality end product. It is therefore not surprising that genetic engineering strategies to reduce levels of off-flavours have garnered much interest. Undesirable compounds in lager beer include vicinal diketones (e.g. diacetyl (butter) and pentadione (honey)), dimethyl sulfide (DMS, cooked or rotten vegetables) and hydrogen sulfide (H_2S, rotten eggs). The vicinal diketone diacetyl (2,3-butanedione), one of the main off-flavours in lager-style beers, is formed extracellularly by spontaneous decarboxylation of α-acetolactate, an intermediate of the valine and leucine biosynthetic pathways in yeast (Krogerus & Gibson, 2013). Another vicinal diketone, 2,3-pentanedione, is similarly derived from isoleucine biosynthesis. This compound and has a honey-like flavour that is undesirable in most beer types. Its human sensory threshold (HST) level (1.5 mg L^{-1}) is ten-fold higher than that of diacetyl (0.15 mg L^{-1}). A low-temperature lagering period after fermentation, during which the yeast reduces diacetyl to acetoin and 2,3-butanediol, and 2,3-pentanedione to 2,3-pentanediol (Figure 1.3) is required to reduce vicinal diketone concentrations. This lagering step is essential for flavour improvement, but has a significant negative impact on the time and energy requirements of industrial brewing.

To accelerate beer maturation, brewers may add a purified acetolactate decarboxylase enzyme (brand name Maturex (Novozyme)). In addition, several genetic engineering strategies have been investigated to lower the formation of diacetyl, with most focusing on the intermediate α-acetolactate. Amino acid biosynthesis pathways in yeast have been explored as targets for reducing vicinal diketone formation. Deletion of *ILV2*, which encodes acetolactate synthase, leads to reduced diacetyl formation, but causes growth defects due to an inability to synthesize amino acids valine, leucine, and isoleucine (Petersen *et al.*, 1983, Gjermansen *et al.*, 1988). Modulating the promoter sequence of *ILV2* reduces its expression level and limits diacetyl formation, while generating enough enzyme activity for amino acid biosynthesis (Petersen *et al.*, 1983). Additionally, upregulating the branched-chain amino acid biosynthesis pathway downstream of α-acetolactate by overexpressing *ILV5*, successfully reduces total vicinal diketone formation without compromising cell growth (Omura, 2008). Deletion of *ILV6*, which encodes a regulatory subunit of acetolactate synthase, reduces activity of *ILV2* and diminishes diacetyl formation (Duong *et al.*, 2011).

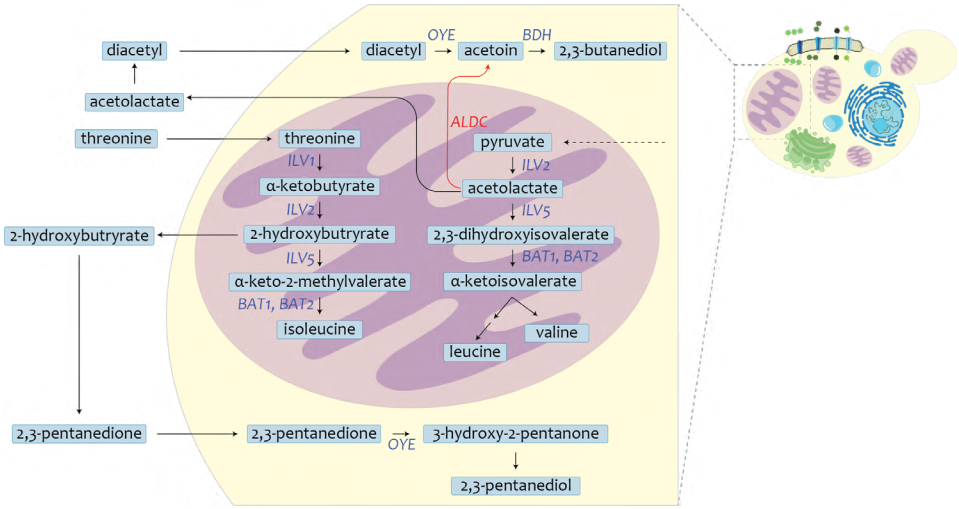


Figure 1.3. Schematic representation of vicinal diketone off-flavour production from the isoleucine, leucine and valine (ILV) biosynthesis pathways in brewer's yeasts. Key reactions in amino acid biosynthesis occur in the mitochondria. The intermediates 2-hydroybutyrate and acetolactate are transported over the (mitochondrial) membrane, after which spontaneous decarboxylation results in formation of 2,3-pentanedione and diacetyl, respectively. Genes encoding enzymes responsible for relevant conversions are highlighted in blue.

Some prokaryotes and Archaea harbour enzymes that catalyse a direct conversion of α-acetolactate to acetoin. Expression of such a heterologous α-acetolactate decarboxylase from *Brevibacillus brevis*, *Enterobacter aerogenes*, *Aerobacter aerogenes*, *Acetobacter aceti ssp. Xylinum*, *Bacillus subtilis* or *Leuconostoc lactis* in *S. cerevisiae* or *S. pastorianus* significantly reduces diacetyl formation (Sone *et al.*, 1988, Fujii *et al.*, 1990, Suihko *et al.*, 1990, Blomqvist *et al.*, 1991, Yamano *et al.*, 1994, Yamano *et al.*, 1995, Guo *et al.*, 2001, **Chapter 4**) and remains the most efficient metabolic engineering strategy for reducing diacetyl formation to date.

Saccharomyces yeast can convert hydroxycinnamic acids present in wort, of which ferulic acid is the most abundant, into phenolic off-flavour molecules. Of these POF compounds, vinyl guaiacol (4VG), with its spicy clove-like flavour, is most problematic as its sensory threshold is very low (0.3 mg L^{-1}) (McMurrough *et al.*, 1996, Krogerus *et al.*, 2016, Krogerus *et al.*, 2017, Yu *et al.*, 2001) (Figure 1.4). Such phenolic compounds are considered off-flavours in most beers, with the exception of some Belgian beers and German Hefeweizen beers. The *S. cerevisiae* genome harbours two genes that are essential for the POF^+ phenotype. *FDC1* encodes the responsible decarboxylase and *PAD1* its essential co-factor. These adjacent genes are located on chromosome IV (Clausen *et al.*, 1994, Mukai *et al.*, 2010). The POF^+ phenotype is highly strain specific and mostly lost (POF^-) in domesticated brewing strains (Baker *et al.*, 2015, Gallone *et al.*, 2016, Gonçalves *et al.*, 2016). In Frohberg (group II) *S. pastorianus* strains, the *SeFDC1-SePAD1* gene cluster has often been lost due to translocations of the ScCHRIV

and the *Se*CHRXIII arms, while the *S. cerevisiae Sc*FDC1 contains a frameshift causing a premature stop codon and *Sc*PAD1 has two non-synonymous mutations (Nakao *et al.*, 2009, van den Broek *et al.*, 2015, Salazar *et al.*, 2019). High-throughput mutagenesis, screening and selection strategies have been developed to generate POF^- brewing strains (Mertens *et al.*, 2015) and POF^- *S. eubayanus* isolates (Diderich *et al.*, 2018). Also, targeted engineering using CRISPR-Cas9 has been exploited to generate a standardized methodology for systematic introduction of a naturally occurring mutation in *FDC1* into *S. cerevisiae* × *S. eubayanus* hybrids (Mertens *et al.*, 2019).

Undesirable sulfides can impart off-flavours to beer when their concentration exceeds the human sensory threshold (100 µg L^{-1}) and, thereby, significantly affect the beer's flavour stability and consistency. One such off-flavour is dimethyl-sulfide (DMS), a by-product of the mashing and fermentation processes. S-methylmethionine (SMM) is the precursor for DMS and originates from methionine formed during germination and kilning of barley in the malting process. Although relevant genetic factors are largely unknown, the high-affinity permease encoded by *MMP1* transports SMM into the cell after which it is metabolized to methionine by a methyl transferase. A premature stop codon in the *MMP1* transporter gene ($MMP1^{G536A}$) has been identified in wine and brewing yeasts. The resulting truncated protein of 179 instead of 583 amino acids unlikely to be functional, which could well lead to reduced SMM uptake by yeast cells (Eder *et al.*, 2022). Another target for reduction of DMS formation is the gene *MRX1*, which encodes a methionine sulfoxide reductase involved in conversion of DMSO to DMS (Hansen *et al.*, 2002).

Hydrogen sulfide (H_2S) adversely affects the flavour of lager beer. H_2S can be formed by yeast-mediated reduction of sulfate to sulfide, resulting in characteristic a rotten egg smell (Figure 1.4). Both minimizing the generation of H_2S and increasing the synthesis of homocysteine, which involves incorporation of H_2S, have been investigated in metabolic engineering studies. Deleting *MET10*, which encodes sulfite reductase (Hansen & Kielland-Brandt, 1996) and overexpression of *CYS4* encoding cystathionine β-synthase were both shown to significantly reduce the release of H_2S in the final beer (Tezuka *et al.*, 1992).

Improving the production of flavour-active compounds: acetate esters, ethyl esters and higher alcohols

Volatile esters, present in trace amounts, play a crucial role in shaping the flavour profile (Verstrepen *et al.*, 2003, Pires *et al.*, 2014). Esters are formed in yeast metabolism through an enzyme-catalysed condensation reaction between an alcohol and an activated acyl-coenzyme A (acyl-CoA) molecule. The identity of the alcohol and the acyl moiety determine which ester, and therefore which flavour, is formed. The rate of ester biosynthesis is determined by the concentration of these two substrates and and by the kinetics and specificity of the responsible enzyme. Two main groups

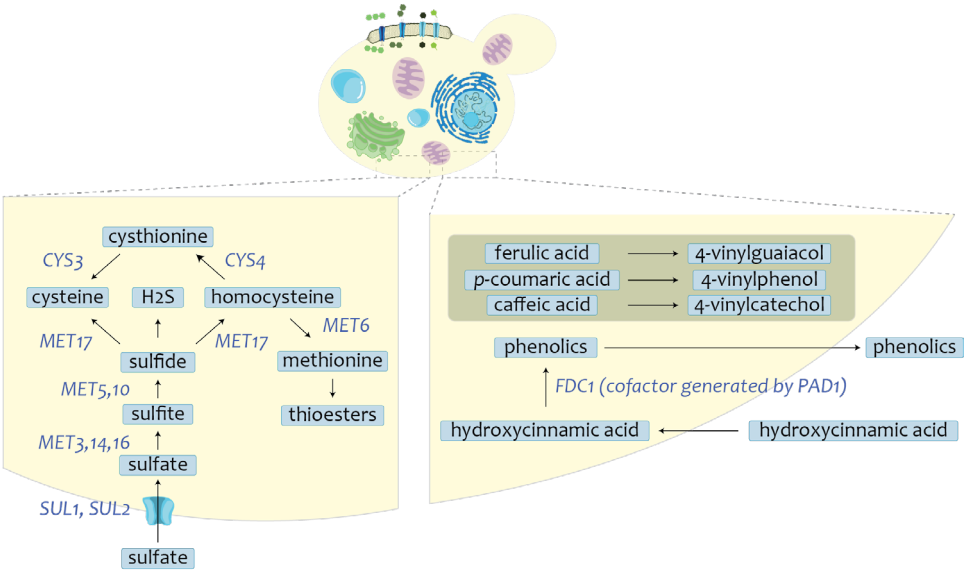


Figure 1.4. Schematic representation of phenolic off-flavour production (right) and sulfur metabolism (left) in brewer's yeasts. Phenolic off-flavours are produced from decarboxylation reactions catalysed by Fdc1, which requires a cofactor generated by Pad1. Sulfate is the main precursor for sulfur-containing off-flavours in beer. Common phenolic off-flavour reactions are highlighted in the green box. Genes encoding enzymes responsible for conversions are highlighted in blue.

of esters can be identified based on their precursor molecules: the acetate esters (e.g., isoamyl acetate, ethyl acetate, isobutyl acetate and phenylethyl acetate) and ethyl esters (e.g., ethyl hexanoate, ethyl octanoate) (Verstrepen *et al.*, 2003) (Figure 1.5). Several hypotheses have been postulated regarding the physiological role of ester biosynthesis. The most pertinent of these is that ester formation contributes to detoxification of higher alcohols and medium chain fatty acids (Nordström, 1964, Hundová & Fencl, 1977, Taylor & Kirsop, 1977, Saerens, 2008, Saerens *et al.*, 2010).

The formation of acetate esters involves acetylation of fusel alcohols. These alcohols originate from the Ehrlich pathway, which decarboxylates and subsequently reduces the carbon skeletons of some amino acids (Vuralhan *et al.*, 2005, Hazelwood *et al.*, 2008, Romagnoli *et al.*, 2012). Alternatively, acetate esters can be formed from ethanol and acetyl-CoA by alcohol acetyltransferases (AATases), encoded by *ATF1* and *ATF2* (Fujii *et al.*, 1994, Fujii *et al.*, 1996, Yoshimoto *et al.*, 1998, Yoshimoto *et al.*, 1999, Mason & Dufour, 2000, Verstrepen *et al.*, 2003) (Figure 1.5). Ethyl acetate, an ester of ethanol and acetyl-CoA with a solvent-like flavour, is synthesized by the AAT enzyme encoded by *EAT1* (Kruis *et al.*, 2017, Kruis *et al.*, 2018). Ethyl esters are formed by the condensation of ethanol and an acyl-CoA molecule, of which the carbon chain may vary from 2 to 10. Acyl-CoA and ethanol are converted to their ethyl ester derivative by acyl-coenzyme A:ethanol O-acyltransferase esterases (AEAT) encoded by *EEB1*

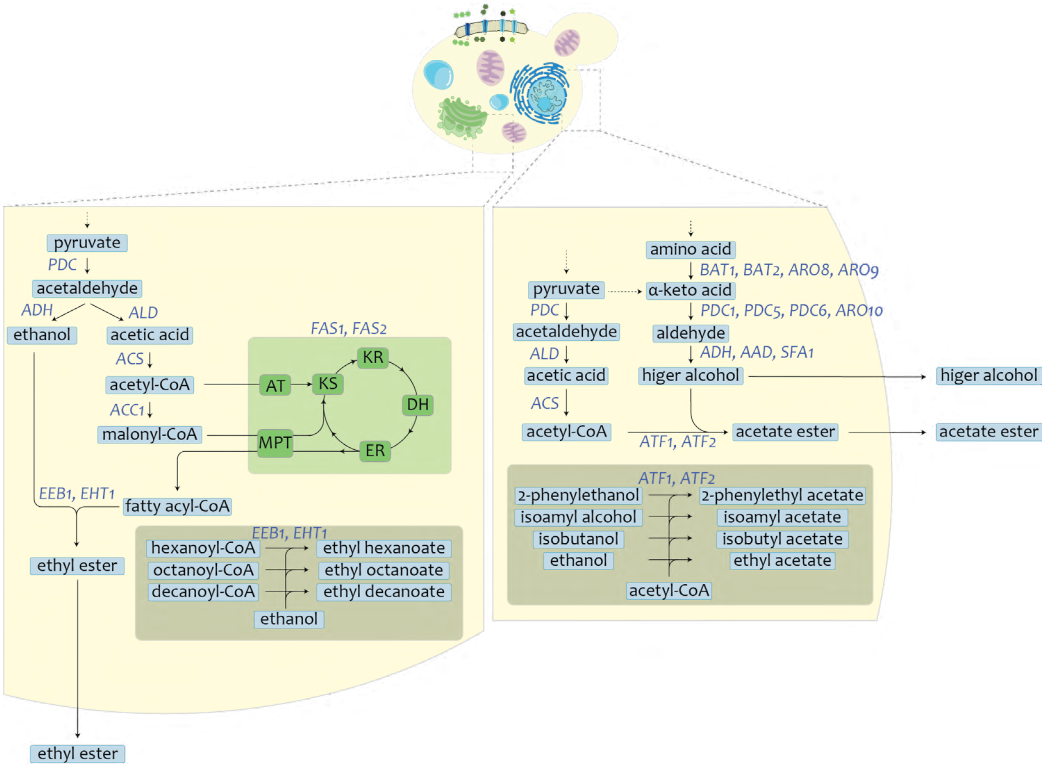


Figure 1.5. Schematic representation of ethyl ester (left) and acetate ester (right) biosynthesis in brewer's yeasts. Ethyl esters are synthesized from fatty acyl-CoA molecules that are released during fatty acid biosynthesis by the Fas1/Fas2 fatty-acid synthase complex (highlighted in light green). Fatty acid synthesis starts with acetyl-CoA and increases the acyl-CoA chain in cyclic reactions with malonyl-CoA moieties. Condensation of the released fatty acyl-CoA with ethanol catalysed by *EEB1* and *ETH1*-encoded enzymes results in ester formation. Acetate esters are produced from acetylation of ethanol or higher alcohols with acetate catalysed by Atf1 and Atf2. Common esters are highlighted in the green box. Genes encoding enzymes responsible for conversions are highlighted in blue.

(Saerens *et al.*, 2006) and *EHT1* (Mason & Dufour, 2000, Saerens *et al.*, 2006, Knight *et al.*, 2014), with Eeb1p being the primary enzyme responsible for this conversion (Saerens *et al.*, 2006) (Figure 1.5). Intriguingly, overexpressing the esterases *EEB1* and *EHT1* in *S. cerevisiae* does not in itself result in a large increase in ethyl ester levels, indicating that the precursor concentration rather than esterase activity may be the limiting factor for ester formation, (Saerens *et al.*, 2006, Saerens, 2008, Saerens *et al.*, 2008). Recently, a study overexpressing *EEB1*, encoding the esterase, and *ETR1*, which encodes a mitochondrial 2-enoyl thioester reductase involved in fatty acid metabolism in yeast, did show increased levels of ethyl hexanoate and ethyl octanoate, which however remained below human sensory threshold levels (Yin *et al.*, 2019).

Other flavours whose production levels have been targets for genetic engineering include higher alcohols (isoamyl alcohol, 2-phenylethanol, propanol, butanol, and isobutanol) (Pires *et al.*, 2014). Higher alcohols are synthesized via the Ehrlich pathway. In this pathway, amino acids are transaminated into the respective α-keto acids by specific transaminases encoded by *BAT1*, *BAT2*, *ARO8* or *ARO9*. After the transamination, the α-keto acid is decarboxylated to the corresponding aldehyde by decarboxylases encoded by *PDC1*, *PDC5*, *PDC6* or *ARO10*, with each having its preferred substrate. Subsequently, the resulting 'fusel aldehydes' are reduced to the corresponding higher alcohols alcohol dehydrogenases encoded by *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5* and *ADH6* (Romagnoli *et al.*, 2012). Several genes, including *ARO80*, encode regulators that control and balance the biosynthesis of higher alcohols in the cell. In addition to the Ehrlich pathway (e.g. 2-phenylethanol from phenylalanine), fusel alcohols (e.g. 2-phenylethanol) can also be synthesized *de novo* from phosphoenolpyruvate and erythose-4-phosphate via the shikimate pathway and the Ehrlich pathway (Hassing *et al.*, 2019) (Figure 1.6). Deregulation of genes involved in the amino acid biosynthesis pathway, shikimate pathway and Ehrlich pathway has resulted in increased fusel alcohol levels (Bolat *et al.*, 2013, Pires *et al.*, 2014, Dzialo *et al.*, 2017, Hassing *et al.*, 2019).

Improvement of beer production efficiency and profitability

In addition to enhancing the quality of the final beer, brewers also seek to increase the brewing efficiency in terms of yield, rate and titre. These objectives often align with the development of a more sustainable production process. One example is the reduction or elimination of the off-flavour diacetyl, as the lagering period after the fermentation that is required to reduce its concentration has a significant impact on the time and energy requirements of the beer production process (Krogerus & Gibson, 2013). Zooming in on the fermentation, the logical approach is to strive for a more efficient use of available nutrients (e.g. sugars and nitrogen compounds) by yeast. The fermentable sugar content of wort in decreasing order of abundance consists of maltose, maltotriose, glucose, sucrose and fructose. Yeast strains generally prefer monosaccharides, followed by disaccharides and only then trisaccharides which, in

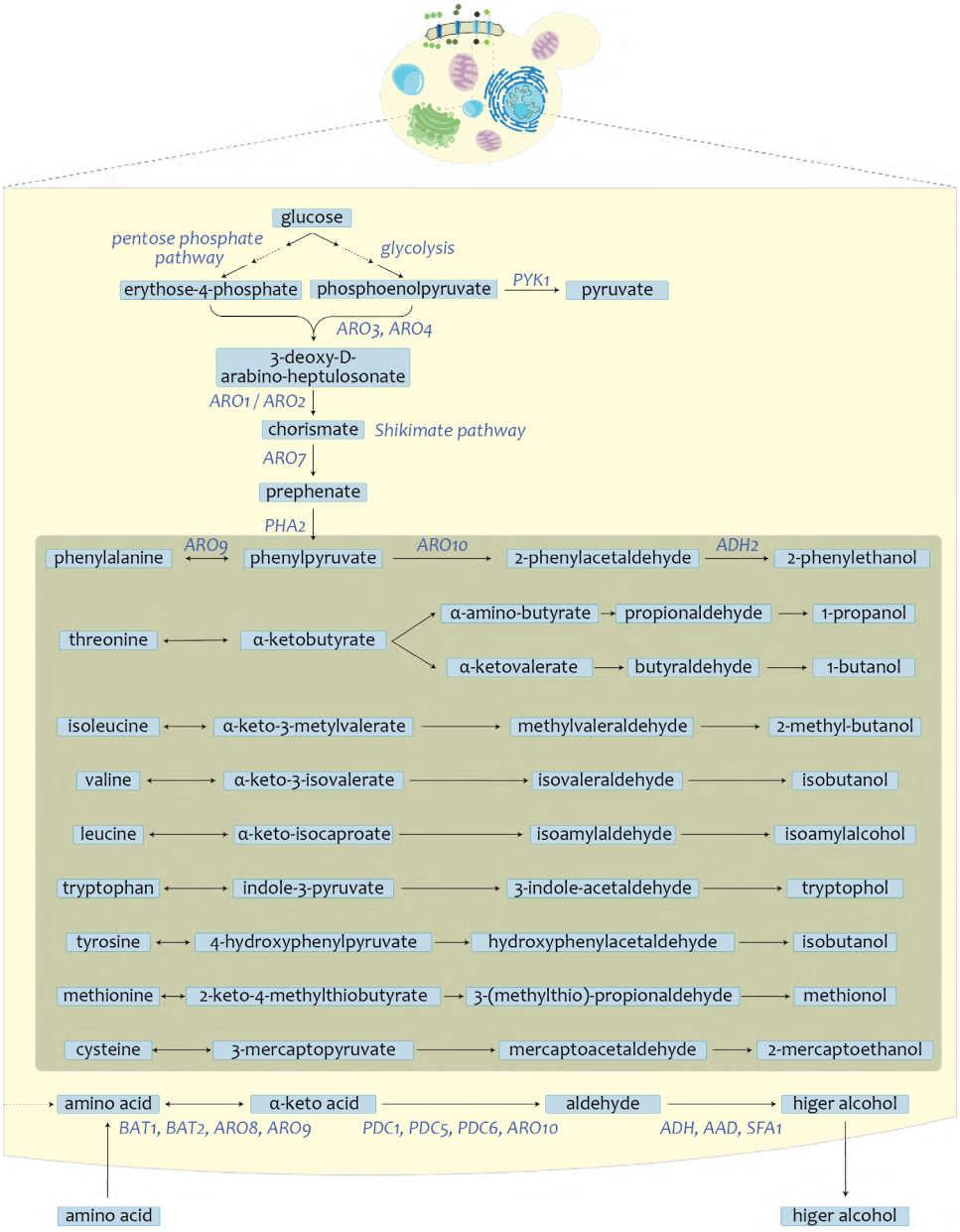


Figure 1.6. Schematic representation of higher alcohol biosynthesis in brewer's yeasts. Higher alcohols are synthesized from branched-chain amino acids that are transaminated to yield the corresponding α-keto acids (transaminase encoded by *BAT1*, *BAT2*, *ARO8* or *ARO9*), followed by decarboxylation to the aldehydes (decarboxylases encoded by *PDC1*, *PDC5*, *PDC6* or *ARO10*) and reduction towards the higher alcohols (alcohol dehydrogenases encoded by *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5* and *ADH6*). Phenyl ethanol can be derived from the amino acid phenylalanine. Alternatively, they can be formed from phenylpyruvate, synthesized via the shikimate and Ehrlich pathway. Specific higher alcohol synthesis routes are highlighted in the green box. Genes encoding enzymes responsible for conversions are highlighted in blue.

many strains, are incompletely fermented. Lower final sugar concentrations change flavour by reducing sweetness, and simultaneously, ethanol production is increased.

Sedimentation of yeast cells in the final stage of fermentation, which involves the aggregation of yeast cells in a process called flocculation, is genetically complex. The exact contribution of different flocculins, which are encoded by a set of *FLO* genes, to flocculation phenotypes remains unknown, as well the impact of external factors on *FLO* gene activity (e.g. stress factors, ethanol concentrations, nutrient concentrations, temperature, pH agitation, wort aeration and calcium concentration) (Watari *et al.*, 1994, Verstrepen *et al.*, 2001, Verstrepen *et al.*, 2003, Vidgren & Londesborough, 2011). The timing and efficiency of flocculation are essential for the beer production efficiency and genetic engineering could be a valuable method for achieving controlled biomass separation during fermentation.

Alleviating the rate liming step in sugar metabolism: membrane transport

The efficient utilization of fermentable sugars in wort requires transport across the membrane by transmembrane transporter proteins. In *Saccharomyces* yeasts, facilitated diffusion of hexoses is mediated by a set of up to 20 different hexose transporters with different kinetic properties (Boles & Hollenberg, 1997). In contrast, disaccharide and trisaccharide transport is facilitated by proton symporters that rely on energy supplied by the electrochemical proton gradient over the membrane. The uptake of maltose and maltotriose is considered to be the rate-limiting step in their metabolism, making these transmembrane transporters highly relevant genetic engineering targets (Kodama *et al.*, 1995). In *S. cerevisiae*, the metabolism of maltose and the associated *MAL* genes are extensively characterized in terms of sequence, genetics, regulation, and biochemistry. The *MAL* loci in *S. cerevisiae* contain three genes that encode proteins with essential roles in maltose metabolism: a transcriptional regulator (*MALx3 S. cerevisiae* and *MALRx* in *S. eubayanus*), a maltose transporter (*MALx1* in *S. cerevisiae* and *MALTx* in *S. eubayanus*) and a maltase (*MALx2 S. cerevisiae* and *MALSx* in *S. eubayanus*) (Needleman, 1991, Jespersen *et al.*, 1999, Vidgren *et al.*, 2005, Brickwedde *et al.*, 2018). *MAL* loci are typically located in subtelomeric regions, with the structurally identical *MAL1*, 2, 3, 4, and 6 located near telomeres of CHRVII, III, II, XI, and VIII, respectively (Charron *et al.*, 1989) (Figure 1.7).

S. cerevisiae MALx1 genes share high sequence similarity (100% for $MAL21_{CEN.PK113\text{-}7D}$ and $MAL31_{CEN.PK113\text{-}7D}$, 75% for $MAL41_{CEN.PK113\text{-}7D}$ compared to *MAL21* and *MAL31* at the nucleotide level), and encode proton symporters with a high affinity for maltose (K_m 2-4 mM) and cannot transport maltotriose (Alves *et al.*, 2008, Brickwedde *et al.*, 2017). The exception is *MAL11* (also known as *AGT1*), which encodes the Mal11/Agt1 transporter, which has only 57% sequence identity to other *MALx1* genes (Han *et al.*, 1995, Vidgren *et al.*, 2009). Mal11 has a much broader substrate range than the other Malx1 transporters (Han *et al.*, 1995, Day *et al.*, 2002, Vidgren *et al.*, 2005, Alves *et al.*, 2008, Vidgren *et al.*, 2009), that

includes maltose, maltotriose (both with K_m values of 20-35 mM), sucrose and trehalose (Day *et al.*, 2002). *MAL* genes are repressed in the presence of glucose. In contrast to *S. cerevisiae*, the *S. eubayanus* type strain CBS 12357 originating from Patagonia is able to metabolise maltose, but no growth is observed on maltotriose as sole carbon source. Investigation of the *SeMALT* genes revealed maltose transport functionality for all the encoded proteins, with *SeMALT2* and *SeMALT4* being predominantly expressed (Brickwedde *et al.*, 2018).

The hybrid *S. pastorianus* yeasts inherited *MAL* genes from both their parental species, resulting in two allelic forms corresponding to *S. cerevisiae* and *S. eubayanus* counterparts. Although *S. pastorianus* has inherited most of its *MALx1* (and *AGT1*) genes from *S. cerevisiae* and *MALT* from *S. eubayanus* (Brickwedde *et al.*, 2018), not all sequences homologous to these ancestral genes encode functional transporters. Non-functionality is often due to frame shift mutations (e.g. *ScAGT1*$^{-1182T}$ (Vidgren *et al.*, 2005, Alves *et al.*, 2008, Nakao *et al.*, 2009, Vidgren *et al.*, 2009, Vidgren *et al.*, 2010, Vidgren & Londesborough, 2012, Cousseau *et al.*, 2013, van den Broek *et al.*, 2015)) or early stop codons (e.g. *ScMAL41*^{298TGA}, *SeMALT*^{259TAA} and *SeMALT3*^{694TGA} (Salazar *et al.*, 2019)). Simply overexpressing *MAL* transporter genes in *S. cerevisiae* brewing stains has been shown to increase their maltose fermentation rate (Kodama *et al.*, 1995). Restoring the *ScAGT1* gene functionality resulted in improved maltose and maltotriose fermentation kinetics (Vidgren *et al.*, 2009). The observation that the *S. cerevisiae AGT1* allele is usually non-functional has led to the hypothesis that a lager-type *AGT1* originating from the *S. eubayanus* was responsible for maltotriose uptake in *S. pastorianus* (Vidgren *et al.*, 2010, Vidgren & Londesborough, 2012, Cousseau *et al.*, 2013). Indeed, similarities between the *S. eubayanus AGT1* genes present in the Himalayan and Holarctic isolates and *S. cerevisiae ScAGT1* were found (Bing *et al.*, 2014, Hebly *et al.*, 2015, Peris *et al.*, 2016, Baker & Hittinger, 2019). Paradoxically, Asian *S. eubayanus* isolates are unable to metabolize maltose and maltotriose, despite harbouring a seemingly intact *SeAGT1* and *SeMALT1* genes. This inability has been attributed to the absence of the required activator gene (Brouwers *et al.*, 2019). Remarkably, the *SeAGT1* gene (85% similarity to *ScAGT1* (Vidgren & Londesborough, 2012)) present in *S. pastorianus* strains typically encodes a functional maltose- and maltotriose-proton symporter (Vidgren & Londesborough, 2012, Cousseau *et al.*, 2013). The activity of the *SeAGT1* gene is thought to result from cross-talk of regulatory genes and transporter genes of the different subgenomes that restores the lack of transcriptional regulation of *SeAGT* observed in the Himalayan *S. eubayanus* (Vidgren & Gibson, 2018, Brouwers *et al.*, 2019). This phenomenon is an example of heterosis, or hybrid vigour.

In addition to *AGT1*/*MAL11*, some lager yeasts strains have an additional transporter gene called *MTT1* or *MTY1*. These genes are not found in *S. cerevisiae* or *S. eubayanus* has and encode transporters with a higher affinity for maltotriose (K_m 16-27 mM) than for maltose (K_m 61-88 mM) (Dietvorst *et al.*, 2005, Salema-Oom *et al.*, 2005, Vidgren *et*

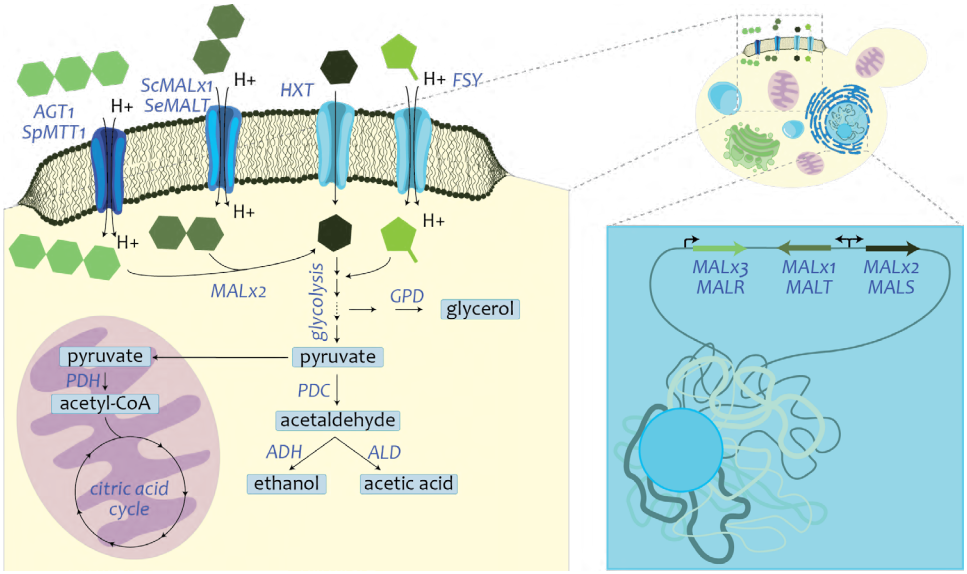


Figure 1.7. Schematic representation of sugar transport and metabolism in brewer's yeasts. Glucose is imported via facilitated diffusion through transmembrane proteins encoded by HXT genes. Fructose, maltose and maltotriose are transported into the cell via proton-symporters encoded by *FSY1*, *ScMALx1*, *SeMALT*, *AGT1* and *SpMTT1*. Oligosaccharides are hydrolysed into glucose monomers that enter the glycolysis whose product pyruvate can be metabolized to ethanol or be converted into biosynthetic precursors by the citric acid cycle. Genes involved in malto(trio)se metabolism are clustered in MAL loci that have a canonical structure of a bi-directional promoter between *MALx1*/*MALT* genes encoding transporters and *MALx2*/*MALS* genes encoding glucosidases that is activated by regulators encoded by *MALx3*/*MALR*. Genes encoding enzymes responsible for conversions are highlighted in blue.

al., 2009, Vidgren & Londesborough, 2012, Cousseau *et al.*, 2013, Magalhães *et al.*, 2016). It is hypothesized that *MTT1* is the result of recombination between *MAL* transporter genes, as sequence comparisons revealed that *MTT1* has a high sequence similarity (92%) to *ScMAL31* (Cousseau *et al.*, 2013), but also has new domains with similarities to genes from *S. paradoxus*, likely resulting from introgressions (Vidgren *et al.*, 2009, Brouwers *et al.*, 2019, Brouwers *et al.*, 2019). This phenomenon has also been observed in laboratory evolution experiments, where intergenic recombination of transporter genes specific for maltose have led to the emergence of chimeric transporter genes with the ability to transport maltotriose (Baker & Hittinger, 2019, Brouwers *et al.*, 2019). Hypothetically, *MTT1* could have emerged in a similar manner due to domestication of *S. pastorianus* under brewing conditions. *MTT1* has been overexpressed in laboratory ale strains, however, its ability to transport maltotriose was not tested (Vidgren *et al.*, 2010). In 2016, the *MTT1* gene was also identified in ale yeasts, thereby questioning the hypothesized *S. eubayanus* origin of *MTT1* (Magalhães *et al.*, 2016). The presence and number of *SpMTT1* copies varies in Saaz and Frohberg strains (Magalhães *et al.*, 2016).

1

Advances in brewing innovation

Unlocking flavour potential: biotransformation of hop-derived molecules

In recent years, craft breweries have engaged in creating distinctive and innovative beers, with emphasis on unique flavour profiles. Adjusting flavour profiles often involves altering the absolute and relative quantities of yeast metabolism-derived compounds, rather than introducing entirely new flavour compounds. Additionally, to brew alternative flavour-enriched beers, brewers are exploring diverse adjuncts, including fruits, spices, herbs, and various grains. There is also an emerging interest in utilising yeasts to enzymatically modify hop-derived molecules during fermentation. Examples of such yeast-mediated biotransformation reactions include the release of polyfunctional thiols from cysteine- or glutathione-bound precursors and the release of terpene alcohols from conjugated glucosides. Both these biotransformations enhance the fruity and floral characteristics of the beverage already at extremely low concentrations of the resulting flavour compounds (HST in the ng L^{-1} range). The key enzyme facilitating thiol release is a cysteine-S-conjugate β-lyase encoded by the *S. cerevisiae IRC7* gene. This enzyme, which is in the native yeast sulfur metabolism and amino acid biosynthesis (Dzialo *et al.*, 2017, Buiatti *et al.*, 2023), releases thiols such as 3-mercaptohexan-1-ol (3MH, grapefruit flavour), 4-mercapto-4-methylpentan-2-one (4MMP, black currant) and 3-sulfanyl-4-methyl-pentan-1-ol (3S4MP, grapefruit) from the cysteinylated and glutathionylated precursors (Molitor *et al.*, 2022, Svedlund *et al.*, 2022). The thiol 3MH can undergo further processing by the *ATF1*-encoded acetyl transferase, yielding the acetate ester 3-mecaptohexyl acetate (3MHA, guava and passion fruit flavour) (Molitor *et al.*, 2022). The β-lyase activity varies among brewing strains due to genetic variations, including a 38 bp deletion ($IRC7^{S}$) or inactivation mutations ($IRC7^{T185A}$) in the *IRC7* gene (Holt *et al.*, 2018, Krogerus *et al.*, 2021, Svedlund *et al.*, 2022). Hybridization of *Saccharomyces* species with varying β-lyase activity followed by screening and selection has yielded brewing strains with increased volatile thiol release (Krogerus *et al.*, 2023)

Recent research has highlighted the complexity of hop composition, influenced by genetics, maturity, and farming practices and climate. Together, these factors have a major influence on the terpene composition of hops (Lafontaine *et al.*, 2021). Terpenes and terpene alcohols (e.g. linalool, geraniol), which are major contributors to the floral, citrus and fruity flavours of the beer, exist in hop oils as the free compounds, but also as glycoside-bound forms with glucose, galactose, xylose, rhamnose or glucuronic acids as sugar(-like) residues. In glycosylated form, the aroma compounds are more soluble and less reactive. Glycosyltransferases combine activated sugar with aglycose compounds (in this case the terpene), while, conversely, β-glucosidase activity hydrolyses the terpene-sugar bonds (Svedlund *et al.*, 2022, Buiatti *et al.*, 2023). Similarly to β-lyase activity for thiol release, β-glucosidase activities vary significantly

among yeast strains (Sharp *et al.*, 2017). Several commercial enzyme preparations, including Aromazyme (Lallemand), Rapidase (DSM-Firmenich) or Sumizyme (Takabio) can be added to increase levels of volatile hop-derived flavour compounds. In addition to expanding the flavour palette, utilising biotransformation reactions can enhance the sustainability of the production process by reducing the need for raw materials, especially hops, which have a large environmental impact due to land use and water requirements.

De novo biosynthesis of non-native flavour molecules

Genetic engineering can not only be used to modify the concentrations of compounds that are naturally produced by yeasts, but also to expand the repertoire of molecules that can be made during fermentation. The ale yeast *S. cerevisiae* has been successfully engineered to produce the terpene molecules geraniol and linalool, which are commonly extracted from hops (Denby *et al.*, 2018). Yeast naturally produces sesquiterpenes (e.g. squalene) from the precursors GPP and FFP, synthesized via the tightly regulated mevalonate pathway (Ro *et al.*, 2006). Heterologous expression of linalool and geraniol synthase genes from basil and mint plants allowed for the conversion of GPP to the non-native terpenes linalool and geraniol, respectively. Combined overexpression of a truncated version of yeast's *HMGR* gene encoding HMG-CoA reductase, lacking the regulatory domain thus alleviating feedback regulation, and introduction of a FFP synthase mutant with reduced activity, increases the GPP precursor supply, and pushes the flux towards the terpene products (Denby *et al.*, 2018).

To circumvent the need for extensive genetic engineering in an industrial brewing strains, which are less amenable to genetic modification, co-fermentation of a laboratory *S. cerevisiae* strain engineered for the production of O-methyl anthranilate providing grape flavour, with the industrial ale strain WLP644, caused a strong change in flavour profile (De Ruijter *et al.*, 2023). Feedback alleviation (*ARO4*^{K229L}, *TRP2*^{S76L}), gene overexpression (*TRP3*, *ARO1*, *ARO2*, *TLK1* and *GLN1*), down regulation (*CDC19*) and deletion (*TRP4*) in the shikimate pathway and aromatic amino acid synthesis pathways combined with heterologous expression of the anthranilic acid methyltransferase 1 gene from *Medicago truncatula* (*MtAAMT1*) in a laboratory *S. cerevisiae* strain, resulted in overproduction of O-methyl anthranilate (400 mg L^{-1}) when cultivated in rich media. Co-fermentation with different strain ratios allowed to produce beers ranging from 60 mg L^{-1} (50% WLP644) to 10 mg L^{-1} (90% WLP644) of this flavour compound (De Ruijter *et al.*, 2023). Difficulties with this co-fermentation setup arise when processes involve recycling of yeasts in consecutive fermentation processes ('repitching'). Engineering the genomes of industrial brewing yeasts could circumvent such challenges, but requires improvement of their genetic accessibility.

Health-based engineering targets: non-alcoholic, calorie-restricted and allergen-free beers

As part of societal trend towards increased health consciousness, there is a growing demand for low and non-alcoholic beverages. Processes for de-alcoholising beer involve significant investments and often result in the loss of esters and higher alcohols. Brewing research is therefore exploring genetic engineering to reduce ethanol content, for example by shifting sugar metabolism towards glycerol production (Nevoigt *et al.*, 2002), or utilizing non-conventional yeast species (e.g. *Saccharomycodes ludwigii*, *Pichia kluyveri*, *Starmerella bombicola*, *Candida pulcherrima*, *Cyberlindnera saturnus*, *Cyberlindnera jadinii*, *Cyberlindnera subsufficiens*, *Saprochaete suaveolens, ZygoSaccharomyces rouxii* and *Torulaspora delbrueckii*) for low-alcohol brewing processes (Canonico *et al.*, 2016, Bellut *et al.*, 2018, Bellut *et al.*, 2019, Adamenko *et al.*, 2020, Tan *et al.*, 2021, Krogerus *et al.*, 2022, Methner *et al.*, 2022, Vaštík *et al.*, 2022). In 2023, Chr. Hansen launched three *Pichia kluyveri* strains called NEER®, NEER® Poly and NEER® Punch. *Pichia kluyveri* only consumes monosaccharides and does not ferment under aerobic conditions. These characteristics limit ethanol production, while yeast-mediated flavour synthesis in the presence of oxygen still confers sensory quality to low alcohol and alcohol-free beers.

In addition to reducing alcohol content, the carbohydrate content of beer can be decreased to yield low-calorie products. Dextrins, which comprise approximately 25% of the sugars in wort, are generally not fermented by brewer's yeasts. While dextrins contribute to the "fullness" or "body" of beer, they also contain a large fraction of its calorie content. Among *Saccharomyces* yeasts, *S. cerevisiae var. diastaticus* is able to produce extracellular glucoamylase enzymes encoded by *STA1*, *STA2* and *STA3* that hydrolyse dextrins into fermentable tri-, di- and monosaccharides (Perry & Meaden, 1988, Sakai *et al.*, 1989). *STA1* was found to be a fusion of *SGA1*, which encodes an intracellular glucoamylase active during sporulation, and *FLO11*, a flocculation protein localized in the cell wall. The *SGA1*-derived part of the protein releases glucose monomers from the dextrin polymer, while the *FLO11*-derived sequence ensures secretion of the enzyme (Krogerus & Gibson, 2020). It was recently discovered that a 1162 bp deletion in the promoter sequence of *STA1* determines the dextrin-hydrolysing capacity of *S. cerevisiae var. diastaticus* strains (Krogerus *et al.*, 2019). Expression of heterologous *STA* genes from fungi belonging to the *Aspergillus* phylum resulted in reduced dextrin content and, as a consequence, higher ethanol content (Cole *et al.*, 1988).

A future perspective in the brewing market could involve production of a zero- or low-alcohol health-promoting beer type. For example, strains of *S. cerevisiae var. boulardii* that have been clinically proven to reduce diarrhea, were investigated for use in production of an alcohol-free beverage with probiotic properties (Senkarcinova *et al.*,

2019). Genetic modification strategies could also be applied to enable brewing gluten-free beers for consumers suffering from celiac disease. Currently, two options are used for the production of gluten-free beer: i) changing the raw materials to cereals other than barley (e.g. corn or rice), and ii) degrading the gluten during the brewing process. The latter approach involves addition of microbial peptidases and, in particular, the prolyl endopeptidase from *Aspergillus niger*. Overexpression of gluten-degrading peptidases in brewer's yeasts could hypothetically degrade gluten peptides, which are generally longer than eight amino acids and rich in proline and glutamine.

Options for improvement of the final beer product as well as the beer production process by the use of genetic modification are tremendously broad. The ongoing developments in brewing strain design and engineering have to the potential to achieve different beer types on demand that comply to consumer demands, are more sustainable, contain innovative flavour profiles, meet personal dietary requirements and are healthier, or even combinations of these.

1.6. Optimization of brewing yeasts: classical engineering strategies

To facilitate changes to traditional brewer's yeast, various close-to-nature methodologies have been applied (Iattici *et al.*, 2020). In a sense, the domestication of brewing yeasts, over many centuries, represented a close-to-natural interplay of microbiological and process factors. Empirically and without awareness of the underlying biology and biochemistry, brewers applied selective pressure by adjusting the brewing conditions and continued with yeast strains that produced the best beer. The resulting domestication yielded yeast strains with distinct phenotypes, including improved low-temperature performance, enhanced sugar utilization, and reduced off-flavour production.

The simplest strategy for strain development programmes in the brewing industry involves exploiting existing biodiversity, which encompasses a range of pure or mixed cultures (Steensels *et al.*, 2014, Steensels & Verstrepen, 2014, Gamero *et al.*, 2016, Gibson *et al.*, 2017, Krogerus *et al.*, 2022) (Figure 1.8A). Often, strains and even species used in industry are chosen for historical reasons rather than based on scientific knowledge (Steensels *et al.*, 2014). New 'wild' yeast isolates can contain adaptations generated through sexual reproduction, mutations, transposons, chromosomal copy number variations, horizontal gene transfers and genetic recombination. However, wild yeasts seldomly possess the phenotypic characteristics required for the process conditions employed in industrial brewing.

After the discovery of *S. eubayanus* as parental species of *S. pastorianus* (Libkind *et al.*, 2011), the brewing potential of the newly discovered species was explored. A limited edition beer was brewed with the Patagonian *S. eubayanus* type strain CBS

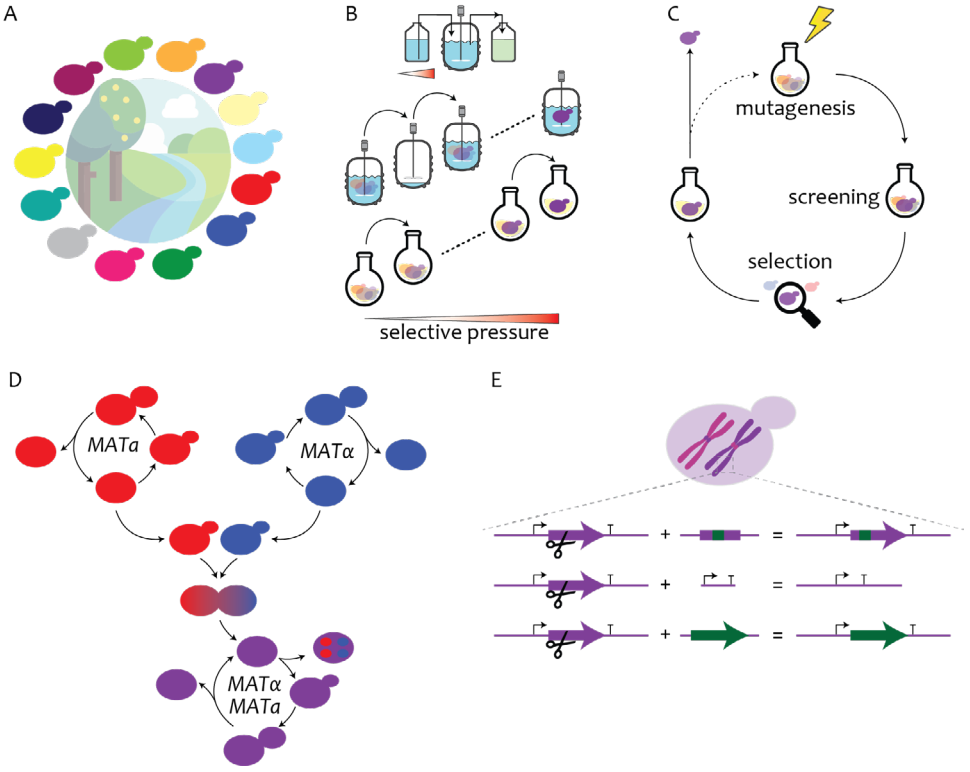


Figure 1.8. Overview of strategies to obtain yeast strains with improved brewing-related phenotypes. **A)** Exploration of natural genetic diversity to find strains with relevant phenotypes, **B)** laboratory evolution, **C)** mutagenesis, screening and selection cycles and **D)** intra- or interspecific hybridization all aim to create genetic variation in natural manner. **E)** Introduction of targeted genetic modifications, including point mutations, gene deletions and integration of expression cassettes for non-native genes at specific genomic locations.

12357 (Libkind *et al.*, 2011) and commercialized by Heineken under the name 'wild lager H41'. Using non-*Saccharomyces* yeasts, which is quite common in the wine industry, is still very rare in industrial beer brewing. A notable successful example is the use of *Brettanomyces* species for production of lambic-type beer styles (Steensels *et al.*, 2015). The use of non-conventional yeasts species may require changes in the fermentation conditions (e.g. oxygen availability, mixing regimes, lower starting Plato) (Cubillos *et al.*, 2019).

Evolutionary engineering, also known as adaptive laboratory evolution, has been extensively applied to obtain variants of yeast strains that outperform their parental strains with respect to industrially relevant aspects of their physiology (Mans *et al.*, 2018) (Figure 1.8B). Laboratory evolution in essence rests on the principle of evolution by natural selection (Darwin, 1859). During cultivation, microorganisms undergo random genetic changes, a small fraction of which may confer a selective advantage (i.e. a higher growth or survival rate) under the experimental conditions.

Depending on the strain and the environment, the natural single-nucleotide mutation rate of *S. cerevisiae* is estimated at $1*10^{-10}$ to $7*10^{-10}$ (Gibson *et al.*, 2020). This mutation rate can be increased with physical and chemical mutagens such as UV radiation or chemicals. In a brewing context, laboratory evolution with *S. pastorianus* grown on increasing concentrations of maltotriose yielded strains with improved maltotriose fermentation kinetics (Brickwedde *et al.*, 2017). In another example, cultivation of a Group I *S. pastorianus* strain in the presence of chlorsulfuron, a compound that inhibits acetolactate synthase, yielded a strain with reduced diacetyl formation (Gibson *et al.*, 2018). In another laboratory evolution experiment, the problem of premature yeast flocculation was tackled by selecting cells that remained in suspension rather dan sedimented cells in a serial transfer experiment with batch cultures (He *et al.*, 2022). Many additional evolutionary engineering strategies have been applied, resulting in improved stress tolerance, increased production of flavour-active molecules, increased sugar utilization and improved flocculation capacities (Ekberg *et al.*, 2013, Conjaerts & Willaert, 2017, Gibson *et al.*, 2020).

The combination of mutagenesis, screening and selection is another 'classical' strain improvement strategy. In this strategy, a cell population is first mutagenized, after which, thousands of mutants are screened for one or more phenotypic traits (Figure 1.8C). Following this strategy, *S. pastorianus* strains with increased osmotolerance were obtained after mutagenesis and enriched on sorbitol-supplemented medium (Ekberg *et al.*, 2013), POF^- *S. eubayanus* were obtained through mutagenesis and screening using a colour reaction assay (Diderich *et al.*, 2018) and mutagenized *S. pastorianus* isolates with improved fermentation performance on very high gravity wort were screened and selected (Huuskonen *et al.*, 2010). Currently, whole genome sequencing can be performed cost-effectively to identify the genetic alteration(s) responsible for the improved characteristic. The main disadvantage of random mutagenesis is that mutations occur at (multiple) random locations in the genome. Consequently, randomly inducing mutations can lead to unintended genetic changes that affect robustness, vitality, or other relevant characteristics.

Yeast hybridization is a strategy that explores genetic diversity by combining genomes of different strains or species (Anderson & Martin, 1975, Steensels *et al.*, 2014, Hebly *et al.*, 2015, Krogerus *et al.*, 2015, Mertens *et al.*, 2015, Krogerus *et al.*, 2016, Gibson *et al.*, 2017, Krogerus *et al.*, 2017, Krogerus *et al.*, 2017, Winans, 2022) (Figure 1.8D). During mating, spores of opposite mating type (*MATa* and *MATα*) can meet and fuse into a new cell. Haploid cells either sporulate into either *MATa* or *MATα*, depending on the mating type locus, and mate with spores of opposite mating type into a diploid *MATa*/*MATα* cell. A diploid cell (*MATa*/*MATα*) sporulates into four spores with single mating types (*MATa*, *MATa*, *MATα* and *MATα*) that mate upon the encounter of a spore of the opposite mating type, resulting in a diploid *MATa*/*MATα* cell. A problem with this classical strain improvement approach is that industrial brewing yeasts often have a

low sporulation efficiency and viability. Moreover, in line with the species definition for eukaryotes, interspecific hybrid yeasts are often sterile. In rare occasions, di- or polyploid cells can become homozygous for the *MAT* locus due to loss of heterozygosity at the mating type locus (Hiraoka *et al.*, 2000). Even cells of different yeast species are sometimes able to mate in a process called rare mating, i.e. mating with spores from the opposite mating type to generate an alloploid cell that, at least initially, combines the two parental genomes. Rare mating can even be used when a strain is unable to form spores (Winans, 2022). As the frequency of rare mating is low (approximately 10^{-4}), selection using complementary auxotrophic strains, cultivation under specific conditions, and/or staining with fluorescent dyes and fluorescence-assisted cell sorting is applied (Steensels *et al.*, 2014, Fukuda *et al.*, 2016, Krogerus *et al.*, 2017, Gorter de Vries *et al.*, 2019). Hybrids resulting from rare mating often show a reduced genetic stability (Krogerus *et al.*, 2015, Mertens *et al.*, 2015, Krogerus *et al.*, 2017). Genetic engineering strategies targeting the mating system have been utilized to enhance interspecific hybridization in brewing contexts. Inducible overexpression of the *HO* gene prior to hybridization enforces mating type switching in a controlled manner, thus bypassing the need for spontaneous loss of heterozygosity (Strathern *et al.*, 1982, Alexander *et al.*, 2016). Similarly, CRISPR-Cas9 has been exploited to introduce a double-strand break at the *HO* locus for generating aneuploid strains with a single mating type useful for hybridization (Krogerus *et al.*, 2021). Deletion of the *HO* gene resulting in heterothallic strains has been widely used to generate stable haploid cells to improve hybridizations (Molinet *et al.*, 2022).

The discovery of *S. eubayanus* (Libkind *et al.*, 2011) led to strong interest in the generation of new *S. cerevisiae* × *S. eubayanus* 'laboratory hybrids' (Hebly *et al.*, 2015, Krogerus *et al.*, 2015, Mertens *et al.*, 2015, Alexander *et al.*, 2016, Krogerus *et al.*, 2016). Such laboratory hybrids possess great potential value for the brewing industry, as it has been shown that they may ferment faster, possess a broader temperature tolerance range, or produce more diverse flavour compounds than their parent strains. However, the wild *S. eubayanus* isolates possess undesirable attributes (e.g. the POF^+ phenotype and the inability to consume maltotriose), which were changed during the domestication of *S. pastorianus*. By accessing the domesticated *S. eubayanus* subgenome and mating these meiotic segregants with *S. cerevisiae* spore isolates, allotetraploid NovaLager hybrid yeasts (1:3 ratio of *S. eubayanus* : *S. cerevisiae* subgenome) were obtained and commercialized as LalBrew NovaLager™ (Lallemand Brewing) (Turgeon *et al.*, 2021). Also hybrids of other *Saccharomyces* species show potential for utilization in the brewing industry (Nikulin *et al.*, 2018, Gyurchev *et al.*, 2022). Since only a low fraction of the generated hybrids exhibit desired traits, selection of promising candidates requires extensive screening.

1.7. Targeted genetic engineering methods

While classical strain development strategies have been widely employed and enabled the generation of strains that are currently used in industry, the processes involved remain time-consuming and reliant on high-throughput screening methods. Furthermore, consecutive cycles of classical strain improvement can accumulate undesired mutations. Targeted strain engineering aims to introduce genetic modifications precisely and exclusively at the genomic location of interest and leave the rest of the genome unaffected. However, implementing such targeted genetic modifications requires novel technologies, complete, high-quality genome sequences of the target strains and a comprehensive understanding of their physiology (Figure 1.8E).

The simplest method for introducing genetic modification of a yeast is probably the transformation of a yeast cell with a plasmid bearing an expression cassette for a gene with a particular function. Use of plasmids, however, requires application of a constant selective pressure for plasmid maintenance. Industrial applicability of such selectable mobile elements is often limited or even precluded under industrial process conditions. Directly modifying the chromosomal DNA (e.g., by introducing integrations, deletions and/or point mutations) provides higher genetic stability and eliminates the need for applying constant selective pressure (Astola *et al.*, 2023).

Almost all current genome editing approaches for yeasts exploit cellular pathways for repair of DNA damage. In *S. cerevisiae*, the homology directed repair mechanism (HDR) that resolves DSBs by using the genetic information from a DNA template, is more efficient than the error-prone non-homologous end-joining mechanism (Orr-Weaver *et al.*, 1981, Szostak *et al.*, 1983). A DSB activates and recruits the cellular DNA repair machinery at the targeted location. The repair machinery then uses a donor DNA template ('repair fragment') with sequences homologous to those flanking the damaged DNA site for repair, thereby introducing any genomic edit included on the repair fragment. *S. cerevisiae* is extremely good at homologous recombination of short homologous sequences, which is a highly advantageous trait for genetic engineering. In the early stages of genome editing, the natural occurrence of DSBs resulted in a low frequency of recombination events at the intended chromosomal target site (1 in 10^6 - 10^9 cells) (Capecchi, 1989). To increase the HDR frequency for genome editing purposes, a series of endonucleases targeting a defined genomic location was applied. Firstly, mega nucleases (e.g. I-SceI and HO) that recognize long DNA sequences (14 to 40 base pairs) were used to introduce targeted DSBs, but their recognition sequences are rare and need to be introduced into yeast chromosomes to create suitable 'landing pads' for integration (Malkova *et al.*, 1996, Storici *et al.*, 2003, Kuijpers *et al.*, 2013). Next, artificial DNA-binding nucleases, such as zinc finger nucleases (ZFNs) (Kandavelou *et al.*, 2005, Urnov *et al.*, 2005) and transcription activator-like effector

nucleases (TALENs) (Christian *et al.*, 2010), were developed to create DSBs at user-defined DNA sites. However, the DNA recognition domains of artificial nucleases are sequence-dependent and each site-directed genetic modification requires a newly designed nuclease.

The most effective approach to date for introducing targeted DSBs traces its roots back to groundbreaking research on bacterial antiviral defence mechanisms (Mojica *et al.*, 2005, Barrangou *et al.*, 2007, Brouns *et al.*, 2008). Clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems have since been extensively studied for their use in genome editing in various organisms, including yeasts (Jinek *et al.*, 2012, Cong *et al.*, 2013, DiCarlo *et al.*, 2013, Jiang *et al.*, 2013, Mali *et al.*, 2013) (Figure 1.9). The freedom to guide the CRISPR endonuclease to almost any location of interest by designing short (approximately 20 bp) guide sequences makes the CRISPR-Cas systems especially attractive. The endonuclease-programming RNA can be composed of an RNA duplex resulting from the combination of a crRNA and a tracrRNA or composed of a single RNA molecule like for Cas9 and Cas12a, respectively (Jinek *et al.*, 2012). When the spacer encoded in the crRNA complements the protospacer sequence in the target DNA, the endonuclease precisely cleaves the target DNA (Garneau *et al.*, 2010). For effective endonuclease activity, the spacer sequence must be adjacent to a CRISPR-system dependent protospacer adjacent motif (PAM) (Garneau *et al.*, 2010). The development of CRISPR-Cas as genetic engineering tool earned Emmanuelle Charpentier and Jenifer A. Doudna the Chemistry Nobel Prize in 2020, highlighting its enormous value for modern research in biology, biotechnology and medicine.

Natural evolution of prokaryotes yielded a variety of Cas systems, which are classified into groups and subtypes based on their structural features and their preferred molecular targets — DNA, RNA, or proteins (Makarova *et al.*, 2011, Makarova *et al.*, 2015, Koonin *et al.*, 2017, Shmakov *et al.*, 2017, Makarova *et al.*, 2020, Hu *et al.*, 2022). Genome editing mostly relies on single-effector class-2 Cas proteins with a distinctive multidomain structure. The Cas9 protein, which belongs to the class 2, type II Cas proteins was the first to be harnessed for gene editing applications and remains the most widely used Cas protein in editing applications (Jinek *et al.*, 2012) (Figure 1.9A). While initial applications were demonstrated in human cell lines, the Cas9 system has proven effective in a wide range of hosts (Jinek *et al.*, 2012, DiCarlo *et al.*, 2013, Feng *et al.*, 2013, Jiang *et al.*, 2013, Li *et al.*, 2013, Mizuno *et al.*, 2014, Gorter de Vries *et al.*, 2017, Juergens *et al.*, 2018). Class 2 type V CRISPR-Cas systems such as Cas12a can serve as an alternative to Cas9 (Zetsche *et al.*, 2015). Cas12a, also known as Cpf1, was the first type V Cas protein tested in yeast and, due to its different sequence requirements, expanded the toolbox for yeast genome editing (Zetsche *et al.*, 2015, Swiat *et al.*, 2017, Verwaal *et al.*, 2018) (Figure 1.9B).

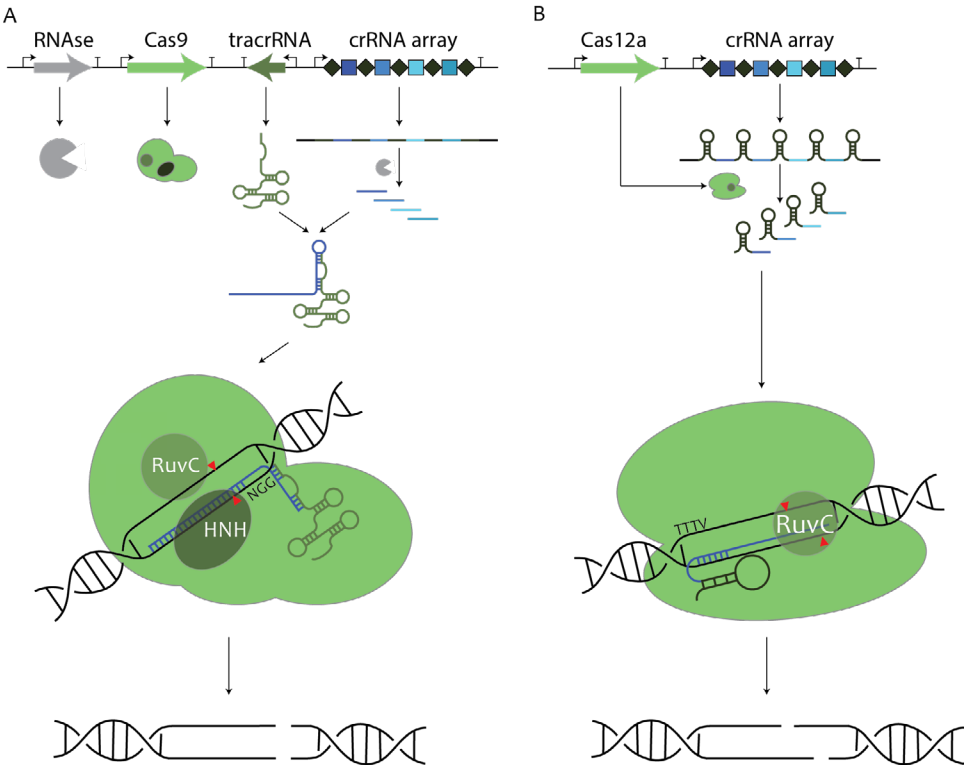


Figure 1.9. Schematic representation of genome editing with **A)** CRISPR-Cas9 and **B)** CRISPR-Cas12a. The premature crRNA array for Cas9 is processed to mature crRNA by RNAse, which forms a complex with the structural tracrRNA. The crRNA array for Cas12a is processed by the intrinsic RNAse activity of Cas12a and releases single mature crRNAs. The tracrRNA-crRNA complex in case of Cas9 and crRNA in case of Cas12a, guides the endonucleases to the protospacers and required PAM sequences (NGG for Cas9 and TTTV for Cas12a). The RuvC and HNH domain of Cas9 cleave the DNA, resulting in blunt ends, whereas the RuvC domain of Cas12a leaves staggered ends upon cleavage.

The widely used *Streptococcus pyogenes* Cas9 (*Spy*Cas9) protein recognizes a 5'-NGG-3' PAM sequence, while the Cas12a proteins recognize T-rich PAM sequences (5'-TTTV-3' or more general 5'-YTTN-3') (Jinek *et al.*, 2012, Swiat *et al.*, 2017). Another difference between the two endonucleases is that Cas12a cleavage occurs distal from the PAM sequence and leaves staggered end cuts, while Cas9 cuts proximal to the PAM and generates blunt ends (Figure 1.9). For efficient application in heterologous hosts, the CRISPR class 2 type II system (Cas9) has been simplified by connecting the crRNA to the tracrRNA in a chimeric single guide RNA (sgRNA), that mimics the natural RNA duplex structure (Jinek *et al.*, 2012). The most interesting feature of Cas12a compared to Cas9, is that the effector module requires neither RNAse nor tracrRNA to process its crRNA (Zetsche *et al.*, 2015) (Figure 1.9). Instead, the Cas12a effector protein recognizes direct repeats in CRISPR arrays and self-processes them to produce single-molecule crRNAs.

After precise site-specific cleavage by the cleavage domain of either Cas9 or Cas12a, the DSB triggers the DNA repair machinery and directs responding proteins to the designated genetic locus. In cells equipped with a functional HDR system, a DNA repair template with terminal sequences homologous to the target site assists in resolving the otherwise lethal DSB, thereby incorporating any desired genetic information between the terminal homology sequences into the genome (Capecchi, 1989).

Genetic engineering using CRISPR-Cas tools was advanced further with the concept of multiplexing (Adiego-Pérez *et al.*, 2019). Combining multiple sgRNAs targeting different genomic locations and supplying DNA repair fragments compatible with each of the target sites, allows for simultaneous implementation of multiple genetic alterations. In this context, using Cas12a has an advantage over Cas9 due to its RNA self-processing ability (Zetsche *et al.*, 2015), which holds the potential to release single crRNAs without the need for RNA cleavage by, for example, flanking ribozymes (e.g. hammerhead or hepatitis delta virus ribozymes), RNase recognizing pre-tRNA sequences or Csy4 sequences, as is required for Cas9 (Gao & Zhao, 2014, He *et al.*, 2017, Ferreira *et al.*, 2018, Zhang *et al.*, 2019). Also, crRNA expressed from constitutive or inducible RNA polymerase II promoters requires post-transcriptional processing, as these transcripts are being capped at the 5' end and extended with a 3'-poly-A-tail (Gao & Zhao, 2014). RNA polymerase III promoters involved in transcription of genes that encode non-mRNA molecules do not perform these post-transcriptional modifications and can be applied without RNA-cleavage strategies (Gao & Zhao, 2014).

Genetic engineering in the hybrid yeast *S. pastorianus*

In the 1980s, the first targeted genetic engineering strategies were applied in *S. pastorianus* (Table 1.1). Introduction of different plasmid-borne expression cassettes conferred a diastatic phenotype and eliminated diacetyl formation. These early methods were soon followed by integrative plasmid strategies and Cre-LoxP-mediated integration procedures (Table 1.1). In these methods, use of (recyclable) genetic markers conferring prototrophy or antibiotic resistance were typically required for stable genetic integration. CRISPR-Cas9-mediated genome engineering has been successfully demonstrated in *S. pastorianus* using ribozyme-flanked gRNAs expressed from RNA polymerase II promoters rather than RNA polymerase III promoters (Gorter de Vries *et al.*, 2017). However, compared to the explosion of genome editing research with CRISPR tools in *S. cerevisiae* (403 hits in PubMed (https://pubmed.ncbi.nlm.nih.gov/) with queries "CRISPR-Cas" AND "*cerevisiae*" on 1st April 2024), the scientific output on CRISPR-based targeted strain engineering in *S. pastorianus* remains low (3 hits in PubMed with queries "CRISPR-Cas" AND "*pastorianus*" on 1st April 2024). The published CRISPR-based genome engineering in *S. pastorianus* currently performed is limited to gene deletions of *SeILV6*, *SeATF1* and *SeATF2* alleles in *S. pastorianus* strains CBS 1483 and WS34/70 (Gorter de Vries *et al.*, 2017) (Table 1.1) and integrations in specific

Table 1.1. Genetic engineering performed in *S. pastorianus*

Genetic modification	Phenotype	Method	Reference
Sugar utilisation			
Aspergillus awamori glaA expression	Increased dextrin utilization	Integrative plasmids with selection markers	(Cole *et al.*, 1988)
S. cerevisiae var. diastaticus DEX overexpression	Increased dextrin utilization	Multicopy plasmid with *CUP1* as selection marker	(Perry & Meaden, 1988)
S. cerevisiae var. diastaticus STA1 overexpression	Increased dextrin utilization	Multicopy plasmid	(Sakai *et al.*, 1989)
AGT1 overexpression	Increased maltose and maltotriose utilization	Integrative plasmids with selection markers	(Vidgren *et al.*, 2009)
Trichoderma reesei EGI1 expression	Increased β-glucan degradation	Multicopy plasmid and integrative plasmid with *CUP1* as selection marker	(Penttilä *et al.*, 1987)
T. reesei EG2, *CBH2* and *BGL1* overexpression	Co-utilisation of xylose and cellulose	Integrative plasmids with *hygR* and *kanMX* as selective markers	(Kricka *et al.*, 2015)
Overexpression *IMA1* and *AGT1*	Utilization of isomaltose and panose	Integrative plasmids in auxotrophic strains	(Porcayo Loza *et al.*, 2021)
LEU1 overexpression	Improved high gravity fermentation	Multicopy plasmid with *kanMX* as selection marker	(Blieck *et al.*, 2007)
Nitrogen utilization			
PUT4 overexpression	Increased proline assimilation	Integration cassette into *URA3*	(Omura *et al.*, 2005)
Alcohol production			
GPD1 overexpression	Increased glycerol production, decreased ethanol production	Multicopy plasmid with *kanMX* as selection marker	(Nevoigt *et al.*, 2002)
Off flavours - diacetyl			
Enterobacter aerogenes aldC overexpression	Bypassing diacetyl formation	Multicopy plasmid	(Sone *et al.*, 1988)
Yeast *ADC1* overexpression	Bypassing diacetyl formation	rDNA integrative plasmid	(Fujii *et al.*, 1990)

Table 1.1. (continued)

Genetic modification	Phenotype	Method	Reference
Enterobacter aerogenes α-ald and *Klebsiella terrigena α-ald* overexpression	Bypassing diacetyl formation	Integrative plasmid with *CUP1* as selection marker	(Suihko *et al.*, 1990)
Enterobacter aerogenes α-ald and *Klebsiella terrigena α-ald* overexpression	Bypassing diacetyl formation	Integrative plasmid with *CUP1* as selection marker	(Blomqvist *et al.*, 1991)
Acetobacter aceti ssp. Xylinum aldC overexpression	Bypassing diacetyl formation	Integrative plasmid into the rRNA region with *kanMX* as selection marker	(Yamano *et al.*, 1994, Yamano *et al.*, 1995)
Bacillus subtilis α-aldC overexpression	Bypassing diacetyl formation	Integrative plasmid with *CUP1* as selection marker	(Guo *et al.*, 2001)
ScILV6 disruption	Decreased diacetyl production	Selection-marker-assisted gene deletion, followed by loxP-mediated marker removal	(Duong *et al.*, 2011)
Deletion of *SeILV6*	Decreased diacetyl formation	CRISPR-Cas9 mediated deletion	(Gorter de Vries *et al.*, 2017)
Off flavours – vicinal diketones			
FDC1 disruption	Decreased 4-vinyl guaiacol production	CRISPR-Cas9 engineering of *S. cerevisiae* prior to hybridization with *S. eubayanus*	(Mertens *et al.*, 2019)
Off flavours – sulfur compounds			
NHS5 overexpression	Decreased H_2S production	Multicopy plasmid	(Tezuka *et al.*, 1992)
MET10 disruption	Increased SO_2	Disruption cassettes containing kanMX selective marker	(Hansen & Kielland-Brandt, 1996)
HOM3 overexpression, *SKP2* disruption	Increased SO_2 and decreased H_2S production	Disruption cassettes containing BSD selective marker	(Yoshida *et al.*, 2008)
Flavour biosynthesis			
ATF1 and *ATF2* overexpression	Increased acetate ester production	Integrative plasmid with *LEU2* as selection marker	(Verstrepen *et al.*, 2003)
Deletion of *SeATF1* and *SeATF2*	Altering ester biosynthesis	CRISPR-Cas9-mediated deletion	(Gorter de Vries *et al.*, 2017)

Table 1.1. (continued)

Genetic modification	Phenotype	Method	Reference
Overexpression *EEB1* and/or *ETR1*	Increased medium-chain ethyl esters	Selection-marker-assisted gene deletion, followed by loxP-mediated marker removal	(Yin *et al.*, 2019)
SeARO80 disruption	Increased higher alcohol production	Selection-marker-assisted gene deletion, followed by loxP-mediated marker removal	(Bolat *et al.*, 2013)
Overexpression of *ATF1*, deletion *BAT2*	Altering the ratio of higher alcohols to esters	Galactose-inducible I-SceI endonuclease for DSB introduction and HR for DSB repair	(Cui *et al.*, 2021)
Other			
URA3 deletion	Uracil auxotroph	Disruption cassettes containing selective markers	(Murakami *et al.*, 2012)
FKS1 disruption	Improved anti-staling of beer due to reduced yeast autolysis	Integrative plasmid with CUP1 as selection marker	(Wang *et al.*, 2014)

locations of the hybrid *Se*Sc*CHRIII* (**Chapter 4**). The complexity of the *S. pastorianus* genome, including its hybrid aneuploid nature, strain-to-strain variation, chimeric chromosomes, inconsistent chromosome copy number variations, and scattered (loss of) heterozygosity, presents challenges for CRISPR-based genetic engineering (Gorter de Vries *et al.*, 2018).

In hybrid yeast strains, gRNAs are not necessarily designed to cut all parental alleles of a target gene due to heterozygosity or allelic variation. Presence of an uncut syntenic homoeologous counterpart of a target sequence may compete with the intended repair DNA fragment as template for homologous recombination. The resulting undesired chromosome recombination can cause loss of heterozygosity, lead to even more undesired genetic changes and significantly reduce editing efficiency. These complications make editing outcomes unpredictable and renders characterization of the edited locus troublesome (Gorter de Vries *et al.*, 2018, Bennis *et al.*, 2023, Jayaprakash *et al.*, 2023). The resulting lack of efficient genome editing tools negatively affects the progress on genetic studies in hybrid, industrial yeasts. Many studies on these yeasts therefore still rely on methods that perform HDR without introduction of a DSB, require the use of selectable markers and can only replace one allele at the time. Innovative solutions are required to accelerate genetic engineering strategies in these complex, hybrid species.

1.8. Current status of the commercial use of genetically engineered yeast in brewing

Strategies for targeted engineering of brewing yeast genomes are predominantly derived from insight gained during biochemical studies or classical strain improvement. Strains resulting from classical strain improvement strategies can more easily be introduced in industry than strains resulting from targeted genetic modification. This difference is related to limited public acceptance of genetically modified organisms (GMOs) and the legislation that governments have implemented in response to those concerns. Since the legalization of Flavr Savr, the first commercially grown genetically engineered food granted a license for human consumption in 1994 by the FDA, regulations regarding genetically engineered food and beverages has not seen drastic changes.

GMO regulations vary significantly across different geographic areas, as exemplified by differences between the USA and EU. The USA has, from the outset, adopted a product-based approach. The FDA categorizes brewing yeasts as processing aid, and mentioning GMO on product labels is not mandatory. In contrast, the EU's GMO legislation is based on the mechanism by which changes are made in the DNA. Recently, the EU is considering shifting from a technology-based to a product- and risk-based evaluation, where the type of DNA modification determines the product's

status. In this context, the use of native DNA or DNA from closely related species (cisgenic) is considered safer than using heterologous (transgenic) DNA. Following a similar line of reasoning, single-nucleotide polymorphisms (SNPs) that might also have been generated by long-accepted non-targeted methods are considered as safe in the USA. Nonetheless, the EU maintains strict regulations, only granting approval for GM foods and beverages after a rigorous safety assessment by the European Food Safety Authority (EFSA).

Despite the complexity surrounding GMO brewing strains at a global scale, yeast producers in the US are enthusiastically generating and commercializing genetically engineered brewing yeasts. Companies like Berkeley Yeast, Lallemand Brewing and Omega Yeast are companies that generate and supply these genetically engineered yeasts, mostly focusing on flavour improvements (Table 1.2).

Table 1.2. Overview of commercially available genetically engineered brewing yeasts.

Commercial name	Organism	Phenotype	Engineering strategy	Source
Diacetyl Free (DF) (includes Chico DF, Hazy Chico DF, London DF and Vermont DF)	*S. cerevisiae*	No diacetyl formation	Heterologous acetolactate decarboxylase (*ALDC*) expression	Berkeley Yeast
Diacetyl free (DF) (includes Fresh DF, Chill DF and Tradition DF)	*S. pastorianus*			Berkeley Yeast
DKO (Diacetyl Knock Out) Series	*S. cerevisiae*			Omega Yeast Labs
CRISPR 5.2%	*S. pastorianus*			Berkeley Yeast
Alexandre Trois	*S. cerevisiae*	Eliminated diastatic fermentation	*STA-1*, encoding glucoseamylase, gene deletion from *Saccharomyces (Brettanomyces) trois* yeast strain	Berkeley Yeast
Sourvisiae ®10	*S. cerevisiae*	Lactic acid production	Heterologous lactate dehydrogenase (*LDH*) expression	Lallemand Brewing
Chico Galactic	*S. cerevisiae*	Lactic acid production	Lactate dehydrogenase (*LDH*) expression	Berkeley Yeast
Tropics (includes London Tropics, Vermont Tropics and Hornindal Tropics)	*S. cerevisiae*	Biotransformation of thiol compounds (3MH, 3MHA and 4MMP)	Expression of carbon-sulphur lyase (CSL) encoded by *tnaA* gene from *E.coli* and alcohol acyl transferase, encoded by *AAT1*	Berkeley Yeast
Tropics (includes Chill Tropics)	*S. pastorianus*			Berkeley Yeast
Cosmic Punch (Thiolized series)	*S. cerevisiae*	Biotransformation of bound thiol compounds (3MH, 3MHA and 4MMP)	Integration of *IRC7* encoding beta-lyase from West Coast I yeast	Omega Yeast Labs

Table 1.2. (continued)

Commercial name	Organism	Phenotype	Engineering strategy	Source
Star Party Ale / Helio Gazer Ale (Thiolized series)	*S. cerevisiae*	Biotransformation of bound thiol compounds (3MH, 3MHA and 4MMP)	Integration of *patB* encoding beta-lyase from bacteria	Omega Yeast Labs
Lunar Crush Lager (Thiolized series)	*S. pastorianus*			Omega Yeast Labs
Chico SuperBloom	*S. cerevisiae*	Terpenoid production (linalool, geraniol and citronellol)	Heterologous expression of *tHMGR* (truncated from yeast), *FPPS* (mutant from yeast), *LIS* (truncated from *M. citrata*) and *GES* (*O. basilicum*)	Berkeley Yeast
Chico Sunburst	*S. cerevisiae*	Ethyl ester production (ethyl butyrate)	Expression of acyl transferase	Berkeley Yeast
Bananza (POF^- series)	*S. cerevisiae*	Banana flavour	*FDC1* gene inactivation (mutation C460T)	Omega Yeast Labs
Sundew (POF^- series)	*S. cerevisiae*	Flavours of strawberry, passion fruit, pear, and stone fruit		Omega Yeast Labs

1

Scope of this thesis

This thesis aims to explore the genetic complexity of the complex hybrid *S. pastorianus* genome with the goal to develop advanced genome-editing strategies to expedite construction of genetically engineered brewing strains exhibiting novel or superior phenotypes relevant for the brewing industry.

The advent of CRISPR-Cas DNA editing technologies has significantly accelerated strain construction in the model yeast *S. cerevisiae*. Several methods have been validated for highly efficient single, and multiplex genome editing events using Cas9 or Cas12a endonucleases. CRISPR-Cas genetic engineering tools rely on target-specific gRNAs for precise genome editing. Each genetic modification requires custom gRNAs, typically cloned into an expression vector prior to transformation into yeast, a process known for its time-consuming and laborious nature. **Chapter 2** introduces a new cloning- and PCR-free methodology for CRISPR-*Fn*Cas12a-meditated editing in *S. cerevisiae* and investigates its efficiency for single and multiplexed editing.

Each CRISPR-endonuclease exhibits unique characteristics, including its PAM sequence, gRNA secondary structure and nucleic-acid cleavage mechanism. **Chapter 3** explores the use of Cas12a isolated from *Eubacterium rectale* as genome editing tool in *S. cerevisiae*. Apart from being of fundamental interest as a genome editing tool in yeasts, *Er*Cas12a is attractive in view of its free commercial research license. The chapter investigates various aspects of *Er*Cas12a and proposes design principles its application in single and multiplexed genome editing in *S. cerevisiae*.

While several studies have used CRISPR-Cas9 for editing the genome of *S. pastorianus*, the lack of a generic, predictable and efficient genetic engineering tool hinders research progress in lager brewing yeast. In particular, the complex aneuploid hybrid genome interferes with CRISPR-Cas9-mediated editing, as introduction of a DSB in a heterozygous location induced unpredictable loss of heterozygosity. **Chapter 4** seeks to address this challenge by identification of unique genomic 'landing sites' devoid of homoeologous regions by exploring their use for integration of expression cassettes, thereby enhancing genetic accessibility of *S. pastorianus*.

In **Chapter 5**, the CRISPR-Cas9 genome editing tool is applied in *S. pastorianus* to increase production of ethyl ester flavour molecules during brewing. By modifying the fatty acid synthase and overexpressing the esterase, the supply of acyl-CoA precursors is increased, leading to increased ethyl ester levels. This study demonstrates that minimal genetic engineering can achieve highly different beer flavour profiles.

1.9. References

Adamenko K, Kawa-Rygielska J & Kucharska AZ (2020) Characteristics of Cornelian cherry sour non-alcoholic beers brewed with the special yeast *Saccharomycodes ludwigii*. *Food Chemistry* **312**: 125968.

Adiego-Pérez B, Randazzo P, Daran JM, Verwaal R, Roubos JA, Daran-Lapujade P & van der Oost J (2019) Multiplex genome editing of microorganisms using CRISPR-Cas. *FEMS Microbiology Letters* **366**.

Aldhous P (1990) Genetic engineering: Modified yeast fine for food. *Nature* **344**: 186.

Alexander WG, Peris D, Pfannenstiel BT, Opulente DA, Kuang M & Hittinger CT (2016) Efficient engineering of marker-free synthetic allotetraploids of *Saccharomyces*. *Fungal Genetics and Biology* **89**: 10-17.

Alves SL, Jr., Herberts RA, Hollatz C, Trichez D, Miletti LC, de Araujo PS & Stambuk BU (2008) Molecular analysis of maltotriose active transport and fermentation by *Saccharomyces cerevisiae* reveals a determinant role for the *AGT1* permease. *Applied Environmental Microbiology* **74**: 1494-1501.

Anderson E & Martin PA (1975) The sporulation and mating of brewing yeasts. *Journal of the Institute of Brewing* **81**: 242-247.

Annaluru N, Muller H, Mitchell LA, *et al.* (2014) Total synthesis of a functional designer eukaryotic chromosome. *Science* **344**: 55-58.

Astola A, Durán-Guerrero E, Díaz AB, Lasanta C & Castro R (2023) Impact of the genetic improvement of fermenting yeasts on the organoleptic properties of beer. *European Food Research and Technology* **249**: 1677-1687.

Baker E, Wang B, Bellora N, Peris D, Hulfachor AB, Koshalek JA, Adams M, Libkind D & Hittinger CT (2015) The genome sequence of *Saccharomyces eubayanus* and the domestication of lager-brewing yeasts. *Molecular Biology and Evolution* **32**: 2818-2831.

Baker EP & Hittinger CT (2019) Evolution of a novel chimeric maltotriose transporter in *Saccharomyces eubayanus* from parent proteins unable to perform this function. *PLOS Genetics* **15**: e1007786.

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA & Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**: 1709-1712.

Bellut K, Michel M, Zarnkow M, Hutzler M, Jacob F, Atzler JJ, Hoehnel A, Lynch KM & Arendt EK (2019) Screening and application of *Cyberlindnera* yeasts to produce a fruity, non-alcoholic beer. *Fermentation* **5**: 103.

Bellut K, Michel M, Zarnkow M, Hutzler M, Jacob F, De Schutter DP, Daenen L, Lynch KM, Zannini E & Arendt EK (2018) Application of non-*Saccharomyces* yeasts isolated from kombucha in the production of alcohol-free beer. *Fermentation* **4**: 66.

Bennis NX, Kostanjšek M, van den Broek M & Daran JG (2023) Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts. *New Biotechnology* **76**: 49-62.

Bergin SA, Allen S, Hession C, Ó Cinnéide E, Ryan A, Byrne KP, Ó Cróinín T, Wolfe KH & Butler G (2022) Identification of European isolates of the lager yeast parent *Saccharomyces eubayanus*. *FEMS Yeast Research* **22**.

Bing J, Han PJ, Liu WQ, Wang QM & Bai FY (2014) Evidence for a Far East Asian origin of lager beer yeast. *Current Biology* **24**: R380-381.

Blieck L, Toye G, Dumortier F, Verstrepen KJ, Delvaux FR, Thevelein JM & Dijck PV (2007) Isolation and characterization of brewer's yeast variants with improved fermentation performance under high-gravity conditions. *Applied and Environmental Microbiology* 73: 815-824.

Blomqvist K, Suihko M-L, Knowles J & Penttilä M (1991) Chromosomal integration and expression of two bacterial α-acetolactate decarboxylase genes in brewer's yeast. *Applied and Environmental Microbiology* **57**: 2796-2803.

Bolat I, Romagnoli G, Zhu F, Pronk JT & Daran JM (2013) Functional analysis and transcriptional regulation of two orthologs of *ARO10*, encoding broad-substrate-specificity 2-oxo-acid decarboxylases, in the brewing yeast *Saccharomyces pastorianus* CBS1483. *FEMS Yeast Research* **13**: 505-517.

Boles E & Hollenberg CP (1997) The molecular genetics of hexose transport in yeasts. *FEMS Microbiology Reviews* **21**: 85-111.

Bond U (2009) Chapter 6: The genomes of lager yeasts. *Advanced Applied Microbiology* **69**: 159-182.

Bond U, Neal C, Donnelly D & James TC (2004) Aneuploidy and copy number breakpoints in the genome of lager yeasts mapped by microarray hybridisation. *Current Genetics* **45**: 360-370.

Boonekamp FJ, Knibbe E, Vieira-Lara MA, Wijsman M, Luttik MAH, van Eunen K, den Ridder M, Bron R, Almonacid Suarez AM, van Rijn P, Wolters JC, Pabst M, Daran JM, Bakker BM & Daran-Lapujade P (2022) Full humanization of the glycolytic pathway in *Saccharomyces cerevisiae*. *Cell Reports* **39**.

Brickwedde A, Brouwers N, van den Broek M, Gallego Murillo JS, Fraiture JL, Pronk JT & Daran J-MG (2018) Structural, physiological and regulatory analysis of maltose transporter genes in *Saccharomyces eubayanus* CBS 12357T. *Frontiers in Microbiology* **9**.

Brickwedde A, van den Broek M, Geertman JA, Magalhães F, Kuijpers NGA, Gibson B, Pronk JT & Daran JG (2017) Evolutionary engineering in chemostat cultures for improved maltotriose fermentation kinetics in *Saccharomyces pastorianus* lager brewing yeast. *Frontiers in Microbiology* **8**: 1690.

Brouns SJJ, Jore MM, Lundgren M, Westra ER, Slijkhuis

RJH, Snijders APL, Dickman MJ, Makarova KS, Koonin EV & van der Oost J (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**: 960-964.

Brouwers N, Gorter de Vries AR, van den Broek M, Weening SM, Elink Schuurman TD, Kuijpers NGA, Pronk JT & Daran J-MG (2019) *In vivo* recombination of *Saccharomyces eubayanus* maltose-transporter genes yields a chimeric transporter that enables maltotriose fermentation. *PLOS Genetics* **15**: e1007853.

Brouwers N, Brickwedde A, Gorter de Vries AR, van den Broek M, Weening SM, van den Eijnden L, Diderich JA, Bai FY, Pronk JT & Daran JG (2019) Himalayan *Saccharomyces eubayanus* genome sequences reveal genetic markers explaining heterotic maltotriose consumption by *Saccharomyces pastorianus* hybrids. *Applied Environmental Microbiology* **85**.

Buiatti S, Tat L, Natolino A & Passaghe P (2023) Biotransformations performed by yeasts on aromatic compounds provided by hop - A review. *Fermentation* **9**: 327.

Caesar R, Palmfeldt J, Gustafsson JS, Pettersson E, Hashemi SH & Blomberg A (2007) Comparative proteomics of industrial lager yeast reveals differential expression of the *cerevisiae* and non-*cerevisiae* parts of their genomes. *Proteomics* **7**: 4135-4147.

Canonico L, Agarbati A, Comitini F & Ciani M (2016) *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. *Food Microbiology* **56**: 45-51.

Capecchi MR (1989) Altering the genome by homologous recombination. *Science* **244**: 1288-1292.

Casaregola S, Nguyen HV, Lapathitis G, Kotyk A & Gaillardin C (2001) Analysis of the constitution of the beer yeast genome by PCR, sequencing and subtelomeric sequence hybridization. *International Journal of Systematic and Evolutionary Microbiology* **51**: 1607-1618.

Charron MJ, Read E, Haut SR & Michels CA (1989) Molecular evolution of the telomere-associated *MAL* loci of *Saccharomyces*. *Genetics* **122**: 307-316.

Cherry JM, Hong EL, Amundsen C, *et al.* (2011) *Saccharomyces* Genome Database: the genomics resource of budding yeast. *Nucleic Acids Research* **40**: D700-D705.

Cho RJ, Campbell MJ, Winzeler EA, Steinmetz L, Conway A, Wodicka L, Wolfsberg TG, Gabrielian AE, Landsman D, Lockhart DJ & Davis RW (1998) A genome-wide transcriptional analysis of the mitotic cell cycle. *Molecular Cell* **2**: 65-73.

Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ & Voytas DF (2010) Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* **186**: 757-761.

Clausen M, Lamb CJ, Megnet R & Doerner PW (1994) PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. *Gene* **142**: 107-112.

Cole GE, McCabe PC, Inlow D, Gelfand DH, Ben-Bassat A & Innis MA (1988) Stable expression of *Aspergillus awamori* glucoamylase in distiller's yeast. *Nature Biotechnology* **6**: 417-421.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA & Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**: 819-823.

Conjaerts A & Willaert RG (2017) Gravity-driven adaptive evolution of an industrial brewer's yeast strain towards a snowflake phenotype in a 3D-printed mini tower fermentor. *Fermentation* **3**: 4.

Cousseau FE, Alves SL, Jr., Trichez D & Stambuk BU (2013) Characterization of maltotriose transporters from the *Saccharomyces eubayanus* subgenome of the hybrid *Saccharomyces pastorianus* lager brewing yeast strain Weihenstephan 34/70. *Letters in Applied Microbiology* **56**: 21-29.

Cubillos FA, Gibson B, Grijalva-Vallejos N, Krogerus K & Nikulin J (2019) Bioprospecting for brewers: Exploiting natural diversity for naturally diverse beers. *Yeast* **36**: 383-398.

Cui D-y, Ge J-l, Song Y-m, Feng P-p, Lin L-c, Guo L-y & Zhang C-y (2021) Regulating the ratio of higher alcohols to esters by simultaneously overexpressing *ATF1* and deleting *BAT2* in brewer's yeast *Saccharomyces pastorianus*. *Food Bioscience* **43**.

Culotti J & Hartwell LH (1971) Genetic control of the cell division cycle in yeast. III. Seven genes controlling nuclear division. *Experimental Cell Research* **67**: 389-401.

Daran-Lapujade P, Daran JM, van Maris AJ, de Winde JH & Pronk JT (2009) Chemostat-based micro-array analysis in baker's yeast. *Advances in Microbial Physiology* **54**: 257-311.

Darwin C (1859) On the origin of species by means of natural selection, or preservation of favoured races in the struggle for life. *London: John Murray*, **1859**.

Day RE, Rogers PJ, Dawes IW & Higgins VJ (2002) Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. *Applied Environmental Microbiology* **68**: 5326-5335.

De Barros Lopes M, Bellon JR, Shirley NJ & Ganter PF (2002) Evidence for multiple interspecific hybridization in *Saccharomyces sensu stricto* species. *FEMS Yeast Research* **1**: 323-331.

De La Cerda Garcia-Caro R, Hokamp K, Roche F, Thompson G, Timouma S, Delneri D & Bond U (2022) Aneuploidy influences the gene expression profiles in *Saccharomyces pastorianus* group I and II strains during fermentation. *PLOS Genetics* **18**: e1010149.

De León-Medina PM, Elizondo-González R, Damas-Buenrostro LC, Geertman JM, Van den Broek M, Galán-Wong LJ, Ortiz-López R & Pereyra-Alférez B (2016) Genome annotation of a *Saccharomyces sp.* lager brewer's yeast. *Genomics Data* **9**: 25-29.

De Ruijter JC, Aisala H, Jokinen I, Krogerus K, Rischer H & Toivari M (2023) Production and sensory analysis of grape flavoured beer by co-fermentation of an industrial and a genetically modified laboratory yeast strain. *European Food Research and Technology* **249**: 1991-2000.

Denby CM, Li RA, Vu VT, Costello, Z. Lin W, Chan LJG, Williams J, Donaldson B, Bamforth CW, Petzold CJ, Scheller HV, Martin HG & Keasling JD (2018) Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer. *Nature Communications* **9**: 965.

DiCarlo JE, Norville JE, Mali P, Rios X, Aach J & Church GM (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research* **41**: 4336-4343.

Diderich JA, Weening SM, van den Broek M, Pronk JT & Daran J-MG (2018) Selection of Pof-*Saccharomyces eubayanus* variants for the construction of *S. cerevisiae* × *S. eubayanus* hybrids with reduced 4-vinyl guaiacol formation. *Frontiers in Microbiology* **9**.

Dietvorst J, Londesborough J & Steensma HY (2005) Maltotriose utilization in lager yeast strains: *MTT1* encodes a maltotriose transporter. *Yeast* **22**: 775-788.

Donalies UE, Nguyen HT, Stahl U & Nevoigt E (2008) Improvement of *Saccharomyces* yeast strains used in brewing, wine making and baking. *Advances in Biochemal Engineering/Biotechnology* **111**: 67-98.

Dujon BA & Louis EJ (2017) Genome diversity and evolution in the budding yeasts (*Saccharomycotina*). *Genetics* **206**: 717-750.

Dunn B & Sherlock G (2008) Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. *Genome Research* **18**: 1610-1623.

Duong CT, Strack L, Futschik M, Katou Y, Nakao Y, Fujimura T, Shirahige K, Kodama Y & Nevoigt E (2011) Identification of *Sc*-type *ILV6* as a target to reduce diacetyl formation in lager brewers' yeast. *Metabolic Engineering* **13**: 638-647.

Dzialo MC, Park R, Steensels J, Lievens B & Verstrepen KJ (2017) Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiology Reviews* **41**: S95-S128.

Eder M, Sanchez I, Camarasa C, Daran J-M, Legras J-L & Dequin S (2022) Genetic bases for the metabolism of the DMS precursor S-methylmethionine by *Saccharomyces cerevisiae*. *Food Microbiology* **106**: 104041.

Ekberg J, Rautio J, Mattinen L, Vidgren V, Londesborough J & Gibson BR (2013) Adaptive evolution of the lager brewing yeast *Saccharomyces pastorianus* for improved growth under hyperosmotic conditions and its influence on fermentation performance. *FEMS Yeast Research* **13**: 335-349.

Feng Z, Zhang B, Ding W, Liu X, Yang DL, Wei P, Cao F, Zhu S, Zhang F, Mao Y & Zhu JK (2013) Efficient genome editing in plants using a CRISPR/Cas system. *Cell Research* **23**: 1229-1232.

Ferreira R, Skrekas C, Nielsen J & David F (2018) Multiplexed CRISPR/Cas9 genome editing and gene regulation using Csy4 in *Saccharomyces cerevisiae*. *ACS Synthetic Biology* **7**: 10-15.

Fortune-Business-Insights (2023) Beer Market. Fortune-Business-Insights, https://www.fortunebusinessinsights.com/beer-market-102489.

Fortune-Business-Insights (2023) Craft Beer Market. Fortune-Business-Insights, https://www.fortunebusinessinsights.com/industry-reports/craft-beer-market-100736.

Fujii T, Kondo K, Shimizu F, Sone H, Tanaka J & Inoue T (1990) Application of a ribosomal DNA integration vector in the construction of a brewer's yeast having α-acetolactate decarboxylase activity. *Applied and Environmental Microbiology* **56**: 997-1003.

Fujii T, Nagasawa N, Iwamatsu A, Bogaki T, Tamai Y & Hamachi M (1994) Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. *Applied Environmental Microbiol ogy* **60**: 2786-2792.

Fujii T, Yoshimoto H, Nagasawa N, Bogaki T, Tamai Y & Hamachi M (1996) Nucleotide sequences of alcohol acetyltransferase genes from lager brewing yeast, *Saccharomyces carlsbergensis*. *Yeast* **12**: 593-598.

Fukuda N, Kaishima M, Ishii J, Kondo A & Honda S (2016) Continuous crossbreeding of sake yeasts using growth selection systems for *a*-type and α-type cells. *AMB Express* **6**: 45.

Gallone B, Mertens S, Gordon JL, Maere S, Verstrepen KJ & Steensels J (2018) Origins, evolution, domestication and diversity of *Saccharomyces* beer yeasts. *Current Opinion in Biotechnology* **49**: 148-155.

Gallone B, Steensels J, Prahl T, *et al.* (2016) Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. *Cell* **166**: 1397-1410.e1316.

Gamero A, Quintilla R, Groenewald M, Alkema W, Boekhout T & Hazelwood L (2016) High-throughput screening of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. *Food Microbiology* **60**: 147-159.

Gao Y & Zhao Y (2014) Self-processing of ribozyme-flanked RNAs into guide RNAs *in vitro* and *in vivo* for CRISPR-mediated genome editing. *Journal Integrative Plant Biology* **56**: 343-349.

Garneau JE, Dupuis MÈ, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH & Moineau S (2010) The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**: 67-71.

Gayevskiy V & Goddard MR (2016) *Saccharomyces eubayanus* and *Saccharomyces arboricola* reside in North Island native New Zealand forests. *Environmental Microbiology* **18**: 1137-1147.

Ghaemmaghami S, Huh W-K, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK & Weissman JS (2003) Global analysis of protein expression in yeast. *Nature* **425**: 737-741.

Giaever G, Chu AM, Ni L, et al. (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**: 387-391.

Gibson B & Liti G (2015) *Saccharomyces pastorianus*: genomic insights inspiring innovation for industry. *Yeast* **32**: 17-27.

Gibson B, Vidgren V, Peddinti G & Krogerus K (2018) Diacetyl control during brewery fermentation via adaptive laboratory engineering of the lager yeast *Saccharomyces pastorianus*. *Journal of Industrial Microbiology and Biotechnology* **45**: 1103-1112.

Gibson B, Geertman JA, Hittinger CT, Krogerus K, Libkind D, Louis EJ, Magalhães F & Sampaio JP (2017) New yeasts-new brews: modern approaches to brewing yeast design and development. *FEMS Yeast Research* **17**.

Gibson B, Dahabieh M, Krogerus K, Jouhten P, Magalhães F, Pereira R, Siewers V & Vidgren V (2020) Adaptive laboratory evolution of ale and lager yeasts for improved brewing efficiency and beer quality. *Annual Review of Food Science and Technology* **11**: 23-44.

Gibson BR, Storgårds E, Krogerus K & Vidgren V (2013) Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the parental species *Saccharomyces eubayanus*. *Yeast* **30**: 255-266.

Gjermansen C, Nilsson-Tillgren T, Petersen JGL, Kielland-Brandt MC, Sigsgaard P & Holmberg S (1988) Towards diacetyl-less brewers' yeast. Influence of ilv2 and ilv5 mutations. *Journal of Basic Microbiology* **28**: 175-183.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H & Oliver SG (1996) Life with 6000 genes. *Science* **274**: 546, 563-547.

Gonçalves M, Pontes A, Almeida P, Barbosa R, Serra M, Libkind D, Hutzler M, Gonçalves P & Sampaio JP (2016) Distinct domestication trajectories in top-fermenting beer yeasts and wine yeasts. *Current Biology* **26**: 2750-2761.

Gorter de Vries AR, Pronk JT & Daran JG (2017) Industrial relevance of chromosomal copy number variation in *Saccharomyces* yeasts. *Applied Environmental Microbiology* **83**.

Gorter de Vries AR, de Groot PA, van den Broek M & Daran J-MG (2017) CRISPR-Cas9 mediated gene deletions in lager yeast *Saccharomyces pastorianus*. *Microbial Cell Factories* **16**: 222.

Gorter de Vries AR, Koster CC, Weening SM, Luttik MAH, Kuijpers NGA, Geertman J-MA, Pronk JT & Daran J-MG (2019) Phenotype-independent isolation of interspecies *Saccharomyces* hybrids by dual-dye fluorescent staining and fluorescence-activated cell sorting. *Frontiers in Microbiology* **10**.

Gorter de Vries AR, Voskamp MA, van Aalst ACA, et al. (2019) Laboratory evolution of a *Saccharomyces cerevisiae* × *S. eubayanus* hybrid under simulated lager-brewing conditions. *Frontiers in Genetics* **10**: 242.

Gorter de Vries AR, Pronk JT & Daran J-MG (2019) Lager-brewing yeasts in the era of modern genetics. *FEMS Yeast Research* **19**.

Gorter de Vries AR, Couwenberg LGF, van den Broek M, de la Torre Cortés P, ter Horst J, Pronk JT & Daran J-MG (2018) Allele-specific genome editing using CRISPR–Cas9 is associated with loss of heterozygosity in diploid yeast. *Nucleic Acids Research* **47**: 1362-1372.

Guo W, He X, Tie C & Zhang B (2001) Expression of α-acetolactate decarboxylase gene from *Bacillus subtilis* in brewer's yeast. *Wei Sheng Wu Xue Bao* **41**: 105-108.

Gyurchev NY, Coral-Medina Á, Weening SM, Almayouf S, Kuijpers NGA, Nevoigt E & Louis EJ (2022) Beyond *Saccharomyces pastorianus* for modern lager brews: Exploring non-*cerevisiae Saccharomyces* hybrids with heterotic maltotriose consumption and novel aroma profile. *Frontiers in Microbiology* **13**.

Han EK, Cotty F, Sottas C, Jiang H & Michels CA (1995) Characterization of *AGT1* encoding a general alpha-glucoside transporter from *Saccharomyces*. *Molecular Microbiology* 17: 1093-1107.

Hansen EC (1883) Recherches sur la physiologie et la morphologie des ferments alcooliques. V. Methodes pour obtenir des cultures pures de *Saccharomyces* et de microorganismes analogous. *Compt Rend Trav Lab Carlsberg* **2**: 92-105.

Hansen EC (1896) Practical studies in fermentation being contributions to the life history of micro-organisms. *E. & F. N. Spon*, London and New York.

Hansen J & Kielland-Brandt MC (1996) Inactivation of *MET10* in brewer's yeast specifically increases SO_2 formation during beer production. *Nature Biotechnology* **14**: 1587-1591.

Hansen J, Bruun SV, Bech LM & Gjermansen C (2002) The level of *MXR1* gene expression in brewing yeast during beer fermentation is a major determinant for the concentration of dimethyl sulfide in beer. *FEMS Yeast Research* **2**: 137-149.

Hartwell LH (1971a) Genetic control of the cell division cycle in yeast. IV. Genes controlling bud emergence

and cytokinesis. *Experimental Cell Research* **69**: 265-276.

Hartwell LH (1971b) Genetic control of the cell division cycle in yeast: II. Genes controlling DNA replication and its initiation. *Journal of Molecular Biology* **59**: 183-194.

Hassing E-J, de Groot PA, Marquenie VR, Pronk JT & Daran J-MG (2019) Connecting central carbon and aromatic amino acid metabolisms to improve *de novo* 2-phenylethanol production in *Saccharomyces cerevisiae*. *Metabolic Engineering* **56**: 165-180.

Hazelwood LA, Daran JM, van Maris AJ, Pronk JT & Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Applied Environmental Microbiology* **74**: 2259-2266.

He Y, Zhang T, Yang N, Xu M, Yan L, Wang L, Wang R & Zhao Y (2017) Self-cleaving ribozymes enable the production of guide RNAs from unlimited choices of promoters for CRISPR/Cas9 mediated genome editing. *Journal of Genetics and Genomics* **44**: 469-472.

He Y, Yin H, Dong J, Yu J, Zhang L, Yan P, Wan X, Hou X, Zhao Y, Chen R, Gibson B & Krogerus K (2022) Reduced sensitivity of lager brewing yeast to premature yeast flocculation via adaptive evolution. *Food Microbiology* **106**: 104032.

Hebly M, Brickwedde A, Bolat I, Driessen MRM, de Hulster EAF, van den Broek M, Pronk JT, Geertman J-M, Daran J-M & Daran-Lapujade P (2015) *S. cerevisiae* × *S. eubayanus* interspecific hybrid, the best of both worlds and beyond. *FEMS Yeast Research* **15**.

Hewitt SK, Donaldson IJ, Lovell SC & Delneri D (2014) Sequencing and characterisation of rearrangements in three *S. pastorianus* strains reveals the presence of chimeric genes and gives evidence of breakpoint reuse. *PLOS ONE* **9**: e92203.

Hiraoka M, Watanabe K, Umezu K & Maki H (2000) Spontaneous loss of heterozygosity in diploid *Saccharomyces cerevisiae* cells. *Genetics* **156**: 1531-1548.

Holmberg S (1982) Genetic differences between *Saccharomyces carlsbergensis* and *S. cerevisiae* II. Restriction endonuclease analysis of genes in chromosome III. *Carlsberg Research Communications* **47**: 233-244.

Holt S, Mukherjee V, Lievens B, Verstrepen KJ & Thevelein JM (2018) Bioflavoring by non-conventional yeasts in sequential beer fermentations. *Food Microbiology* **72**: 55-66.

Hornsey IS (2003) A history of beer and brewing. *Royal Society of Chemistry*, Cambridge, UK.

Hornsey IS (2012) Alcohol and its role in the evolution of human society. *The Royal Society of Chemistry*, Cambridge, UK.

Hu C, van Beljouw SPB, Nam KH, et al. (2022) Craspase is a CRISPR RNA-guided, RNA-activated protease. *Science* **377**: 1278-1285.

Huh W-K, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS & O'Shea EK (2003) Global analysis of protein localization in budding yeast. *Nature* **425**: 686-691.

Hundová Z & Fencl Z (1977) Toxic effects of fatty acids on yeast cells: Dependence of inhibitory effects on fatty acid concentration. *Biotechnology and Bioengineering* **19**: 1623-1641.

Hutzler M, Morrissey JP, Laus A, Meussdoerffer F & Zarnkow M (2023) A new hypothesis for the origin of the lager yeast *Saccharomyces pastorianus*. *FEMS Yeast Research* 23.

Huuskonen A, Markkula T, Vidgren V, Lima L, Mulder L, Geurts W, Walsh M & Londesborough J (2010) Selection from industrial lager yeast strains of variants with improved fermentation performance in very-high-gravity worts. *Applied and Environmental Microbiology* **76**: 1563-1573.

Iattici F, Catallo M & Solieri L (2020) Designing new yeasts for craft brewing: When natural biodiversity meets biotechnology. *Beverages* **6**.

Jayaprakash P, Barroso L, Vajente M, Maestroni L, Louis EJ, Morrissey JP & Branduardi P (2023) CRISPR-Cas9 engineering in the hybrid yeast *Zygosaccharomyces parabailii* can lead to loss of heterozygosity in target chromosomes. *FEMS Yeast Research* **23**.

Jespersen L, Cesar LB, Meaden PG & Jakobsen M (1999) Multiple alpha-glucoside transporter genes in brewer's yeast. *Applied Environmental Microbiol ogy* **65**: 450-456.

Jewison T, Knox C, Neveu V, Djoumbou Y, Guo AC, Lee J, Liu P, Mandal R, Krishnamurthy R, Sinelnikov I, Wilson M & Wishart DS (2012) YMDB: the Yeast Metabolome Database. *Nucleic Acids Research* **40**: D815-820.

Jiang W, Bikard D, Cox D, Zhang F & Marraffini LA (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology* **31**: 233-239.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA & Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816-821.

Jones GM, Stalker J, Humphray S, West A, Cox T, Rogers J, Dunham I & Prelich G (2008) A systematic library for comprehensive overexpression screens in *Saccharomyces cerevisiae*. *Nature Methods* **5**: 239-241.

Juergens H, Varela JA, Gorter de Vries AR, Perli T, Gast VJM, Gyurchev NY, Rajkumar AS, Mans R, Pronk JT, Morrissey JP & Daran J-MG (2018) Genome editing in *Kluyveromyces* and *Ogataea* yeasts using a broad-host-range Cas9/gRNA co-expression plasmid. *FEMS Yeast Research* **18**.

Kandavelou K, Mani M, Durai S & Chandrasegaran S (2005) 'Magic' scissors for genome surgery. *Nature*

Biotechnology **23**: 686-687.
Karabin M, Jelinek L, Kotrba P, Cejnar R & Dostalek P (2018) Enhancing the performance of brewing yeasts. *Biotechnology Advances* **36**: 691-706.
Kirin-Holdings (2022) Global Beer Consumption by Country in 2021. Vol. 2022 KirinHoldings, https://www.kirinholdings.com/.
Knight MJ, Bull ID & Curnow P (2014) The yeast enzyme Eht1 is an octanoyl-CoA:ethanol acyltransferase that also functions as a thioesterase. *Yeast* **31**: 463-474.
Kodama Y, Fukui N, Ashikari T, Shibano Y, Morioka-Fujimoto K, Hiraki Y & Nakatani K (1995) Improvement of maltose fermentation efficiency: constitutive expression of *MAL* genes in brewing yeasts. *Journal of the American Society of Brewing Chemists* **53**: 24-29.
Koonin EV, Makarova KS & Zhang F (2017) Diversity, classification and evolution of CRISPR-Cas systems. *Current Opinion in Microbiology* **37**: 67-78.
Kricka W, James TC, Fitzpatrick J & Bond U (2015) Engineering *Saccharomyces pastorianus* for the co-utilisation of xylose and cellulose from biomass. *Microbial Cell Factories* **14**: 61.
Krogerus K & Gibson BR (2013) 125th Anniversary Review: Diacetyl and its control during brewery fermentation. *Journal of the Institute of Brewing* **119**: 86-97.
Krogerus K & Gibson B (2020) A re-evaluation of diastatic *Saccharomyces cerevisiae* strains and their role in brewing. *Applied Microbiology and Biotechnology* **104**: 3745-3756.
Krogerus K, Rettberg N & Gibson B (2023) Increased volatile thiol release during beer fermentation using constructed interspecies yeast hybrids. *European Food Research and Technology* **249**: 55-69.
Krogerus K, Magalhães F, Vidgren V & Gibson B (2015) New lager yeast strains generated by interspecific hybridization. *Journal of Industrial Microbiology and Biotechnology* **42**: 769-778.
Krogerus K, Magalhães F, Vidgren V & Gibson B (2017) Novel brewing yeast hybrids: creation and application. *Applied Microbiology and Biotechnology* **101**: 65-78.
Krogerus K, Seppänen-Laakso T, Castillo S & Gibson B (2017) Inheritance of brewing-relevant phenotypes in constructed *Saccharomyces cerevisiae* × *Saccharomyces eubayanus* hybrids. *Microbial Cell Factories* **16**: 66.
Krogerus K, Magalhães F, Kuivanen J & Gibson B (2019) A deletion in the *STA1* promoter determines maltotriose and starch utilization in *STA1*$^{+}$ *Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology* **103**: 7597-7615.
Krogerus K, Eerikäinen R, Aisala H & Gibson B (2022) Repurposing brewery contaminant yeast as production strains for low-alcohol beer fermentation. *Yeast* **39**: 156-169.
Krogerus K, Fletcher E, Rettberg N, Gibson B & Preiss R (2021) Efficient breeding of industrial brewing yeast strains using CRISPR/Cas9-aided mating-type switching. *Applied Microbiology and Biotechnology* **105**: 8359-8376.
Krogerus K, Arvas M, De Chiara M, Magalhães F, Mattinen L, Oja M, Vidgren V, Yue JX, Liti G & Gibson B (2016) Ploidy influences the functional attributes of *de novo* lager yeast hybrids. *Applied Microbiology and Biotechnology* **100**: 7203-7222.
Kruis AJ, Levisson M, Mars AE, van der Ploeg M, Garcés Daza F, Ellena V, Kengen SWM, van der Oost J & Weusthuis RA (2017) Ethyl acetate production by the elusive alcohol acetyltransferase from yeast. *Metabolic Engineering* **41**: 92-101.
Kruis AJ, Gallone B, Jonker T, Mars AE, van Rijswijck IMH, Wolkers–Rooijackers JCM, Smid EJ, Steensels J, Verstrepen KJ, Kengen SWM, van der Oost J & Weusthuis RA (2018) Contribution of Eat1 and other alcohol acyltransferases to ester production in *Saccharomyces cerevisiae*. *Frontiers in Microbiology* **9**.
Kuijpers NG, Chroumpi S, Vos T, Solis-Escalante D, Bosman L, Pronk JT, Daran JM & Daran-Lapujade P (2013) One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in *Saccharomyces cerevisiae*. *FEMS Yeast Research* **13**: 769-781.
Kumaran R, Yang S-Y & Leu J-Y (2013) Characterization of chromosome stability in diploid, polyploid and hybrid yeast cells. *PLOS ONE* **8**: e68094.
Kyselová L, Hanzalíková K, Matoulková D & Kubizniaková P (2023) Brewers lost in wild yeast nomenclature. *Kvasny prumysl* **69**: 707-718.
Lafontaine S, Caffrey A, Dailey J, Varnum S, Hale A, Eichler B, Dennenlöhr J, Schubert C, Knoke L, Lerno L, Dagan L, Schönberger C, Rettberg N, Heymann H & Ebeler SE (2021) Evaluation of variety, maturity, and farm on the concentrations of monoterpene diglycosides and hop volatile/nonvolatile composition in five *Humulus lupulus* cultivars. *Journal of Agricultural and Food Chemistry* **69**: 4356-4370.
Langdon QK, Peris D, Eizaguirre JI, Opulente DA, Buh KV, Sylvester K, Jarzyna M, Rodríguez ME, Lopes CA, Libkind D & Hittinger CT (2020) Postglacial migration shaped the genomic diversity and global distribution of the wild ancestor of lager-brewing hybrids. *PLOS Genetics* **16**: e1008680.
Lashkari DA, DeRisi JL, McCusker JH, Namath AF, Gentile C, Hwang SY, Brown PO & Davis RW (1997) Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proceedings of the National Academy of Sciences* **94**: 13057-13062.
Lee MG & Nurse P (1987) Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature* **327**: 31-35.
Legras JL, Merdinoglu D, Cornuet JM & Karst F (2007)

Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. *Molecular Ecology* **16**: 2091-2102.

Li J-F, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM & Sheen J (2013) Multiplex and homologous recombination–mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotechnology* **31**: 688-691.

Libkind D, Hittinger CT, Valério E, Gonçalves C, Dover J, Johnston M, Gonçalves P & Sampaio JP (2011) Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences* **108**: 14539-14544.

Lin CL, García-Caro RdlC, Zhang P, Carlin S, Gottlieb A, Petersen MA, Vrhovsek U & Bond U (2021) Packing a punch: understanding how flavours are produced in lager fermentations. *FEMS Yeast Research* **21**.

Liti G, Peruffo A, James SA, Roberts IN & Louis EJ (2005) Inferences of evolutionary relationships from a population survey of LTR-retrotransposons and telomeric-associated sequences in the *Saccharomyces sensu stricto* complex. *Yeast* **22**: 177-192.

Liu L, Wang J, Rosenberg D, Zhao H, Lengyel G & Nadel D (2018) Fermented beverage and food storage in 13,000 y-old stone mortars at Raqefet Cave, Israel: Investigating Natufian ritual feasting. *Journal of Archaeological Science: Reports* **21**: 783-793.

Lue NF & Kornberg RD (1987) Accurate initiation at RNA polymerase II promoters in extracts from *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences* **84**: 8839-8843.

Magalhães F, Vidgren V, Ruohonen L & Gibson B (2016) Maltose and maltotriose utilisation by group I strains of the hybrid lager yeast *Saccharomyces pastorianus*. *FEMS Yeast Research* 16.

Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, van der Oost J & Koonin EV (2011) Evolution and classification of the CRISPR-Cas systems. *Nature Reviews Microbiology* **9**: 467-477.

Makarova KS, Wolf YI, Alkhnbashi OS, *et al.* (2015) An updated evolutionary classification of CRISPR-Cas systems. *Nature Reviews Microbiology* **13**: 722-736.

Makarova KS, Wolf YI, Iranzo J, *et al.* (2020) Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. *Nature Research* **18**: 67-83.

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE & Church GM (2013) RNA-guided human genome engineering via Cas9. *Science* **339**: 823-826.

Malkova A, Ivanov EL & Haber JE (1996) Double-strand break repair in the absence of *RAD51* in yeast: a possible role for break-induced DNA replication. *Proceedings of the National Academy of Sciences* **93**: 7131-7136.

Mans R, Daran JG & Pronk JT (2018) Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. *Current Opinion in Biotechnology* **50**: 47-56.

Martini AV & Kurtzman CP (1985) Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces sensu stricto*. *International Journal of Systematic and Evolutionary Microbiology* **35**: 508-511.

Martini AV & Martini A (1987) Three newly delimited species of *Saccharomyces sensu stricto*. *Antonie Van Leeuwenhoek* **53**: 77-84.

Martini AV & Martini A (1998) 44 - *Saccharomyces Meyen ex Reess*. The Yeasts (Kurtzman CP & Fell JW, eds.), p.358-371. *Elsevier*, Amsterdam.

Masneuf I, Hansen J, Groth C, Piskur J & Dubourdieu D (1998) New hybrids between *Saccharomyces sensu stricto* yeast species found among wine and cider production strains. *Applied Environmental Microbiology* **64**: 3887-3892.

Mason AB & Dufour JP (2000) Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* **16**: 1287-1298.

McMurrough I, Madigan D, Donnelly D, Hurley J, Doyle A-M, Hennigan G, McNulty N & Smyth MR (1996) Control of ferulic acid and 4-vinyl guaiacol in brewing. *Journal of the Institute of Brewing* **102**: 327-332.

Mertens S, Steensels J, Saels V, Rouck GD, Aerts G & Verstrepen KJ (2015) A large set of newly created interspecific *Saccharomyces* hybrids increases aromatic diversity in lager beers. *Applied and Environmental Microbiology* **81**: 8202-8214.

Mertens S, Gallone B, Steensels J, Herrera-Malaver B, Cortebeek J, Nolmans R, Saels V, Vyas VK & Verstrepen KJ (2019) Reducing phenolic off-flavors through CRISPR-based gene editing of the *FDC1* gene in *Saccharomyces cerevisiae* x *Saccharomyces eubayanus* hybrid lager beer yeasts. *PLOS ONE* **14**: e0209124.

Methner Y, Hutzler M, Zarnkow M, Prowald A, Endres F & Jacob F (2022) Investigation of non-*Saccharomyces* yeast strains for their suitability for the production of non-alcoholic beers with novel flavor profiles. *Journal of the American Society of Brewing Chemists* **80**: 341-355.

Meussdoerffer FG (2009) A comprehensive history of beer brewing. *Handbook of Brewing*, 1-42.

Michel RH, McGovern PE & Badler VR (1992) Chemical evidence for ancient beer. *Nature* **360**: 24-24.

Mizuno S, Dinh TTH, Kato K, Mizuno-Iijima S, Tanimoto Y, Daitoku Y, Hoshino Y, Ikawa M, Takahashi S, Sugiyama F & Yagami KI (2014) Simple generation of albino C57BL/6J mice with G291T mutation in the tyrosinase gene by the CRISPR/Cas9 system.

Mammalian Genome **25**: 327-334.
Mojica FJM, Díez-Villaseñor C, García-Martínez J & Soria E (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution* **60**: 174-182.
Molinet J, Urbina K, Villegas C, Abarca V, Oporto CI, Villarreal P, Villarroel CA, Salinas F, Nespolo RF & Cubillos FA (2022) A *Saccharomyces eubayanus* haploid resource for research studies. *Scientific Reports* **12**: 5976.
Molitor RW, Roop JI, Denby CM, Depew CJ, Liu DS, Stadulis SE & Shellhammer TH (2022) The sensorial and chemical changes in beer brewed with yeast genetically modified to release polyfunctional thiols from malt and hops. *Fermentation* **8**: 370.
Monerawela C & Bond U (2017) Recombination sites on hybrid chromosomes in *Saccharomyces pastorianus* share common sequence motifs and define a complex evolutionary relationship between group I and II lager yeasts. *FEMS Yeast Research* **17**.
Monerawela C & Bond U (2017) Brewing up a storm: The genomes of lager yeasts and how they evolved. *Biotechnology Advances* **35**: 512-519.
Monerawela C & Bond U (2018) The hybrid genomes of *Saccharomyces pastorianus*: A current perspective. *Yeast* **35**: 39-50.
Monerawela C, James TC, Wolfe KH & Bond U (2015) Loss of lager specific genes and subtelomeric regions define two different *Saccharomyces cerevisiae* lineages for *Saccharomyces pastorianus* Group I and II strains. *FEMS Yeast Research* **15**.
Mukai N, Masaki K, Fujii T, Kawamukai M & Iefuji H (2010) *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering* **109**: 564-569.
Murakami N, Miyoshi S, Yokoyama R, Hoshida H, Akada R & Ogata T (2012) Construction of a URA3 deletion strain from the allotetraploid bottom-fermenting yeast *Saccharomyces pastorianus*. *Yeast* **29**: 155-165.
Nakao Y, Kanamori T, Itoh T, Kodama Y, Rainieri S, Nakamura N, Shimonaga T, Hattori M & Ashikari T (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Research* **16**: 115-129.
Naseeb S, James SA, Alsammar H, Michaels CJ, Gini B, Nueno-Palop C, Bond CJ, McGhie H, Roberts IN & Delneri D (2017) *Saccharomyces jurei sp. nov.*, isolation and genetic identification of a novel yeast species from *Quercus robur*. *International Journal of Systematic and Evolutionary Microbiology* **67**: 2046-2052.
Naumov GI, James SA, Naumova ES, Louis EJ & Roberts IN (2000) Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*. *International Journal of Systematic and Evolutionary Microbiology* **5**: 1931-1942.
Naumov GI, Naumova ES, Masneuf I, Aigle M, Kondratieva VI & Dubourdieu D (2000) Natural polyploidization of some cultured yeast *Saccharomyces sensu stricto*: auto- and allotetraploidy. *Systematic and Applied Microbiology* **23**: 442-449.
Naumova ES, Naumov GI, Masneuf-Pomarède I, Aigle M & Dubourdieu D (2005) Molecular genetic study of introgression between *Saccharomyces bayanus* and *S. cerevisiae*. *Yeast* **22**: 1099-1115.
Needleman R (1991) Control of maltase synthesis in yeast. *Molecular Microbiology* **5**: 2079-2084.
Nespolo RF, Villarroel CA, Oporto CI, Tapia SM, Vega-Macaya F, Urbina K, De Chiara M, Mozzachiodi S, Mikhalev E, Thompson D, Larrondo LF, Saenz-Agudelo P, Liti G & Cubillos FA (2020) An Out-of-Patagonia migration explains the worldwide diversity and distribution of *Saccharomyces eubayanus* lineages. *PLOS Genetics* **16**: e1008777.
Nevoigt E, Pilger R, Mast-Gerlach E, Schmidt U, Freihammer S, Eschenbrenner M, Garbe L & Stahl U (2002) Genetic engineering of brewing yeast to reduce the content of ethanol in beer. *FEMS Yeast Research* **2**: 225-232.
Nguyen H-V & Gaillardin C (2005) Evolutionary relationships between the former species *Saccharomyces uvarum* and the hybrids *Saccharomyces bayanus* and *Saccharomyces pastorianus*; reinstatement of *Saccharomyces uvarum* (Beijerinck) as a distinct species. *FEMS Yeast Research* **5**: 471-483.
Nikulin J, Krogerus K & Gibson B (2018) Alternative *Saccharomyces* interspecies hybrid combinations and their potential for low-temperature wort fermentation. *Yeast* **35**: 113-127.
Nilsson-Tillgren T, Gjermansen C, Kielland-Brandt MC, Petersen JGL & Holmberg S (1981) Genetic differences between *Saccharomyces carlsbergensis* and *S. cerevisiae*. Analysis of chromosome III by single chromosome transfer. *Carlsberg Research Communications* **46**: 65-76.
Nordström K (1964) Formation of esters from acids by brewer's yeast. IV. Effect of higher fatty acids and toxicity of lower fatty acids. *Journal of the Institute of Brewing* **70**: 233–238.
Okuno M, Kajitani R, Ryusui R, Morimoto H, Kodama Y & Itoh T (2016) Next-generation sequencing analysis of lager brewing yeast strains reveals the evolutionary history of interspecies hybridization. *DNA Research* **23**: 67-80.
Omura F (2008) Targeting of mitochondrial *Saccharomyces cerevisiae* Ilv5p to the cytosol and its effect on vicinal diketone formation in brewing. *Applied Microbiology Biotechnology* **78**: 503-513.
Omura F, Fujita A, Miyajima K & Fukui N (2005)

Engineering of yeast Put4 permease and its application to lager yeast for efficient proline assimilation. *Bioscience Biotechnology Biochemistry* **69**: 1162-1171.

Orr-Weaver TL, Szostak JW & Rothstein RJ (1981) Yeast transformation: a model system for the study of recombination. *Proceedings of the National Academy of Sciences* **78**: 6354-6358.

Pasteur L (1858) Nouveaux faits concernant l'histoire de la fermentation alcoolique. *Comptes Rendus Chimie* **47**: 1011–1013.

Pasteur L (1876) Études sur la bière': ses maladies, causes qui les provoquent, procédé pour la rendre inaltérable. *Gauthier-Villars*, Paris.

Penttilä ME, Suihko ML, Lehtinen U, Nikkola M & Knowles JKC (1987) Construction of brewer's yeasts secreting fungal endo-ß-glucanase. *Current Genetics* **12**: 413-420.

Peris D, Sylvester K, Libkind D, Gonçalves P, Sampaio JP, Alexander WG & Hittinger CT (2014) Population structure and reticulate evolution of *Saccharomyces eubayanus* and its lager-brewing hybrids. *Molecular Ecology* **23**: 2031-2045.

Peris D, Langdon QK, Moriarty RV, Sylvester K, Bontrager M, Charron G, Leducq J-B, Landry CR, Libkind D & Hittinger CT (2016) Complex ancestries of lager-brewing hybrids were shaped by standing variation in the wild yeast *Saccharomyces eubayanus*. *PLOS Genetics* **12**: e1006155.

Perry C & Meaden P (1988) Properties of a genetically-engineered dextrin-fermenting strain of brewers' yeast. *Journal of the Institute of Brewing* **94**: 64-67.

Petersen JGL, Kielland-Brandt MC, Holmberg S & Nilsson-Tillgren T (1983) Mutational analysis of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*. Mapping of ilv2 andilv5. *Carlsberg Research Communications* **48**: 21-34.

Pires EJ, Teixeira JA, Brányik T & Vicente AA (2014) Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology* **98**: 1937-1949.

Poelmans E & Swinnen JFM (2011) From monasteries to multinationals (and back): A historical review of the beer economy. *LICOS - Centre for Institutions and Economic Performance* **6**: 196-216.

Porcayo Loza J, Chailyan A, Forster J, Katz M, Mortensen UH & Garcia Sanchez R (2021) Improving the utilization of isomaltose and panose by lager yeast *Saccharomyces pastorianus*. *Fermentation* **7**.

Pulvirenti A, Nguyen H, Caggia C, Giudici P, Rainieri S & Zambonelli C (2000) *Saccharomyces uvarum*, a proper species within *Saccharomyces sensu stricto*. *FEMS Microbiology Letters* **192**: 191-196.

Querol A & Bond U (2009) The complex and dynamic genomes of industrial yeasts. *FEMS Microbiology Letters* **293**: 1-10.

Rainieri S, Zambonelli C & Kaneko Y (2003) *Saccharomyces sensu stricto*: Systematics, genetic diversity and evolution. *Journal of Bioscience and Bioengineering* **96**: 1-9.

Rainieri S, Kodama Y, Kaneko Y, Mikata K, Nakao Y & Ashikari T (2006) Pure and mixed genetic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. *Applied Environmental Microbiology* **72**: 3968-3974.

Ro DK, Paradise EM, Ouellet M, *et al.* (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440**: 940-943.

Rodríguez ME, Pérez-Través L, Sangorrín MP, Barrio E & Lopes CA (2014) *Saccharomyces eubayanus* and *Saccharomyces uvarum* associated with the fermentation of *Araucaria araucana* seeds in Patagonia. *FEMS Yeast Research* 14: 948-965.

Romagnoli G, Luttik MA, Kötter P, Pronk JT & Daran JM (2012) Substrate specificity of thiamine pyrophosphate-dependent 2-oxo-acid decarboxylases in *Saccharomyces cerevisiae*. *Applied Environmental Microbiology* **78**: 7538-7548.

Saerens S, Thevelein, J., & Delvaux, F. (2008) Ethyl ester production during brewery fermentation, a review. *De Cooman Luc* **33**: 82–90.

Saerens SM, Delvaux FR, Verstrepen KJ & Thevelein JM (2010) Production and biological function of volatile esters in *Saccharomyces cerevisiae*. *Microbial Biotechnology* **3**: 165-177.

Saerens SM, Delvaux F, Verstrepen KJ, Van Dijck P, Thevelein JM & Delvaux FR (2008) Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Applied and Environmental Microbiology* **74**: 454-461.

Saerens SMG, Duong CT & Nevoigt E (2010) Genetic improvement of brewer's yeast: current state, perspectives and limits. *Applied Microbiology and Biotechnology* **86**: 1195-1212.

Saerens SMG, Verstrepen KJ, Van Laere SDM, Voet ARD, Van Dijck P, Delvaux FR & Thevelein JM (2006) The *Saccharomyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. *Journal of Biological Chemistry* **281**: 4446-4456.

Sakai K, Fukui S, Yabuuchi S, Aoyagi S & Tsumura Y (1989) Expression of the *Saccharomyces diastaticus STA1* gene in brewing yeasts. *Journal of the American Society of Brewing Chemists* **47**: 87-91.

Salazar AN, Gorter de Vries AR, van den Broek M, Brouwers N, de la Torre Cortès P, Kuijpers NGA, Daran J-MG & Abeel T (2019) Chromosome level assembly and comparative genome analysis confirm lager-brewing yeasts originated from a single hybridization. *BMC Genomics* **20**: 916.

Salema-Oom M, Valadão Pinto V, Gonçalves P & Spencer-Martins I (2005) Maltotriose utilization by industrial *Saccharomyces* strains: characterization of

a new member of the alpha-glucoside transporter family. *Applied Environmental Microbiology* **71**: 5044-5049.

Sampaio JP (2018) Microbe Profile: *Saccharomyces eubayanus*, the missing link to lager beer yeasts. *Microbiology* **164**: 1069-1071.

Sampaio JP (2022) *Saccharomyces eubayanus*—a tale of endless mysteries. *FEMS Yeast Research* **22**.

Sampaio JP & Gonçalves P (2008) Natural populations of *Saccharomyces kudriavzevii* in Portugal are associated with oak bark and are sympatric with *S. cerevisiae* and *S. paradoxus*. *Applied Environmental Microbiology* **74**: 2144-2152.

Sato M, Kishimoto M, Watari J & Takashio M (2002) Breeding of brewer's yeast by hybridization between a top-fermenting yeast *Saccharomyces cerevisiae* and a cryophilic yeast *Saccharomyces bayanus*. *Journal of Bioscience and Bioengineering* **93**: 509-511.

Schwann T (1837) Vorläufige Mittheilung, bettreffend Versuche über die Weingährungund Fäulniss. *Annalen der Physik und Chemie* 41: 184-193.

Senkarcinova B, Graça Dias IA, Nespor J & Branyik T (2019) Probiotic alcohol-free beer made with *Saccharomyces cerevisiae var. boulardii*. LWT **100**: 362-367.

Shampay J, Szostak JW & Blackburn EH (1984) DNA sequences of telomeres maintained in yeast. *Nature* **310**: 154-157.

Sharp DC, Steensels J & Shellhammer TH (2017) The effect of hopping regime, cultivar and β-glucosidase activity on monoterpene alcohol concentrations in wort and beer. *Journal of the Institute of Brewing* **123**: 185-191.

Shmakov S, Smargon A, Scott D, et al. (2017) Diversity and evolution of class 2 CRISPR-Cas systems. *Nature Reviews Microbiology* **15**: 169-182.

Smart KA (2007) Brewing yeast genomes and genome-wide expression and proteome profiling during fermentation. *Yeast* **24**: 993-1013.

Sone H, Fujii T, Kondo K, Shimizu F, Tanaka J & Inoue T (1988) Nucleotide sequence and expression of the *Enterobacter aerogenes* α-acetolactate decarboxylase gene in brewer's yeast. *Applied and Environmental Microbiology* **54**: 38-42.

Steensels J & Verstrepen KJ (2014) Taming wild yeast: Potential of conventional and nonconventional yeasts in industrial fermentations. *Annual Review of Microbiology* **68**: 61-80.

Steensels J, Meersman E, Snoek T, Saels V & Verstrepen KJ (2014) Large-scale selection and breeding to generate industrial yeasts with superior aroma production. *Applied and Environmental Microbiology* **80**: 6965-6975.

Steensels J, Snoek T, Meersman E, Nicolino MP, Voordeckers K & Verstrepen KJ (2014) Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiology Reviews* **38**: 947-995.

Steensels J, Daenen L, Malcorps P, Derdelinckx G, Verachtert H & Verstrepen KJ (2015) *Brettanomyces* yeasts - From spoilage organisms to valuable contributors to industrial fermentations. *International Journal of Food Microbiology* **206**: 24-38.

Storici F, Durham CL, Gordenin DA & Resnick MA (2003) Chromosomal site-specific double-strand breaks are efficiently targeted for repair by oligonucleotides in yeast. *Proceedings of the National Academy of Sciences* **100**: 14994-14999.

Strathern JN, Klar AJ, Hicks JB, Abraham JA, Ivy JM, Nasmyth KA & McGill C (1982) Homothallic switching of yeast mating type cassettes is initiated by a double-stranded cut in the *MAT* locus. *Cell* **31**: 183-192.

Suihko ML, Blomqvist K, Penttilä M, Gisler R & Knowles J (1990) Recombinant brewer's yeast strains suitable for accelerated brewing. *Journal of Biotechnology* **14**: 285-300.

Svedlund N, Evering S, Gibson B & Krogerus K (2022) Fruits of their labour: biotransformation reactions of yeasts during brewery fermentation. *Applied Microbiology and Biotechnology* **106**: 4929-4944.

Swiat MA, Dashko S, den Ridder M, Wijsman M, van der Oost J, Daran JM & Daran-Lapujade P (2017) *Fn*Cpf1: a novel and efficient genome editing tool for *Saccharomyces cerevisiae*. *Nucleic Acids Research* **45**: 12585-12598.

Szostak JW, Orr-Weaver TL, Rothstein RJ & Stahl FW (1983) The double-strand-break repair model for recombination. *Cell* **33**: 25-35.

Tamai Y, Momma T, Yoshimoto H & Kaneko Y (1998) Co-existence of two types of chromosome in the bottom fermenting yeast, *Saccharomyces pastorianus*. *Yeast* **14**: 923-933.

Tan M, Caro Y, Shum-Cheong-Sing A, Robert L, François JM & Petit T (2021) Evaluation of mixed-fermentation of *Saccharomyces cerevisiae* with *Saprochaete suaveolens* to produce natural fruity beer from industrial wort. *Food Chemistry* **346**: 128804.

Taylor GT & Kirsop BH (1977) The origin of the medium chain length fatty acids present in beer. *Journal of the Institute of Brewing* **83**: 241-243.

Tezuka H, Mori T, Okumura Y, Kitabatake K & Tsumura Y (1992) Cloning of a gene suppressing hydrogen sulfide production by *Saccharomyces cerevisiae* and its expression in a brewing yeast. *Journal of the American Society of Brewing Chemists* **50**: 130-133.

Turgeon Z, Sierocinski T, Brimacombe CA, Jin Y, Goldhawke B, Swanson JM, Husnik JI & Dahabieh MS (2021) Industrially applicable *de novo* lager yeast hybrids with a unique genomic architecture: creation and characterization. *Applied Environmental Microbiology* **87**.

Urnov FD, Miller JC, Lee Y-L, Beausejour CM, Rock JM,

Augustus S, Jamieson AC, Porteus MH, Gregory PD & Holmes MC (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646-651.

van den Broek M, Bolat I, Nijkamp JF, Ramos E, Luttik MA, Koopman F, Geertman JM, de Ridder D, Pronk JT & Daran JM (2015) Chromosomal copy number variation in *Saccharomyces pastorianus* is evidence for extensive genome dynamics in industrial lager brewing strains. *Applied Environmental Microbiol ogy* **81**: 6253-6267.

Vaštík P, Rosenbergová Z, Furdíková K, Klempová T, Šišmiš M & Šmogrovičová D (2022) Potential of non-*Saccharomyces* yeast to produce non-alcoholic beer. *FEMS Yeast Research* **22**.

Verstrepen KJ, Chambers PJ & Pretorius IS (2006) The development of superior yeast strains for the food and beverage industries: Challenges, opportunities and potential benefits. Yeasts in Food and Beverages, 399-444. *Springer Berlin Heidelberg*, Berlin, Heidelberg.

Verstrepen KJ, Derdelinckx G, Verachtert H & Delvaux FR (2003) Yeast flocculation: what brewers should know. *Applied Microbiology and Biotechnology* **61**: 197-205.

Verstrepen KJ, Derdelinckx G, Delvaux FR, Winderickx J, Thevelein JM, Bauer FF & Pretorius IS (2001) Late fermentation expression of *FLO1* in *Saccharomyces cerevisiae*. *Journal of the American Society of Brewing Chemists* **59**: 69-76.

Verstrepen KJ, Derdelinckx G, Dufour J-P, Winderickx J, Thevelein JM, Pretorius IS & Delvaux FR (2003) Flavor-active esters: Adding fruitiness to beer. *Journal of Bioscience and Bioengineering* **96**: 110-118.

Verstrepen KJ, Van Laere SD, Vanderhaegen BM, Derdelinckx G, Dufour JP, Pretorius IS, Winderickx J, Thevelein JM & Delvaux FR (2003) Expression levels of the yeast alcohol acetyltransferase genes *ATF1*, *Lg-ATF1*, and *ATF2* control the formation of a broad range of volatile esters. *Applied Environmental Microbiology* **69**: 5228-5237.

Verwaal R, Buiting-Wiessenhaan N, Dalhuijsen S & Roubos JA (2018) CRISPR/Cpf1 enables fast and simple genome editing of *Saccharomyces cerevisiae*. *Yeast* **35**: 201-211.

Vidgren V & Londesborough J (2011) 125th Anniversary Review: Yeast flocculation and sedimentation in brewing. *Journal of the Institute of Brewing* **117**: 475-487.

Vidgren V & Londesborough J (2012) Characterization of the *Saccharomyces bayanus*-type *AGT1* transporter of lager yeast. *Journal of the Institute of Brewing* **118**: 148-151.

Vidgren V & Gibson B (2018) Trans-regulation and localization of orthologous maltose transporters in the interspecies lager yeast hybrid. *FEMS Yeast Research* **18**.

Vidgren V, Ruohonen L & Londesborough J (2005) Characterization and functional analysis of the MAL and *MPH* loci for maltose utilization in some ale and lager yeast strains. *Applied Environmental Microbiology* **71**: 7846-7857.

Vidgren V, Multanen JP, Ruohonen L & Londesborough J (2010) The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. *FEMS Yeast Research* **10**: 402-411.

Vidgren V, Huuskonen A, Virtanen H, Ruohonen L & Londesborough J (2009) Improved fermentation performance of a lager yeast after repair of its *AGT1* maltose and maltotriose transporter genes. *Applied Environmental Microbiology* **75**: 2333-2345.

Vuralhan Z, Luttik MA, Tai SL, Boer VM, Morais MA, Schipper D, Almering MJ, Kötter P, Dickinson JR, Daran JM & Pronk JT (2005) Physiological characterization of the *ARO10*-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. *Applied Environmental Microbiology* **71**: 3276-3284.

Walther A, Hesselbart A & Wendland J (2014) Genome sequence of *Saccharomyces carlsbergensis*, the world's first pure culture lager yeast. *G3 Genes|Genomes|Genetics* **4**: 783-793.

Wang J-j, Xu W-n, Li Xe, Li J & Li Q (2014) Absence of fks1p in lager brewing yeast results in aberrant cell wall composition and improved beer flavor stability. *World Journal of Microbiology and Biotechnology* **30**: 1901-1908.

Wang SA & Bai FY (2008) *Saccharomyces arboricolus sp. nov.*, a yeast species from tree bark. *International Journal of Systematic and Evolutionary Microbiology* **58**: 510-514.

Watari J, Nomura M, Sahara H, Koshino S & Keränen S (1994) Construction of flocculent brewer's yeast by chromosomal integration of the yeast flocculation gene *FLO1*. *Journal of the Institute of Brewing* **100**: 73-77.

Wendland J (2014) Lager yeast comes of age. *Eukaryot Cell* **13**: 1256-1265.

Winans MJ (2022) Yeast hybrids in brewing. *Fermentation* **8**: 87.

Winzeler EA, Shoemaker DD, Astromoff A, *et al.* (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**: 901-906.

Yamagishi H & Ogata T (1999) Chromosomal structures of bottom fermenting yeasts. *Systematic and Applied Microbiology* **22**: 341-353.

Yamano S, Tanaka J & Inoue T (1994) Cloning and expression of the gene encoding α-acetolactate decarboxylase from *Acetobacter aceti ssp. xylinum* in brewer's yeast. *Journal of Biotechnology* **32**: 165-171.

Yamano S, Tomizuka K, Sone H, Imura M, Takeuchi T, Tanaka J & Inoue T (1995) Brewing performance of a brewer's yeast having α-acetolactate decarboxylase from *Acetobacter aceti subsp.*

xylinum. *Journal of Biotechnology* **39**: 21-26.

Yin H, Liu L-P, Yang M, Ding X-T, Jia S-R, Dong J-J & Zhong C (2019) Enhancing medium-chain fatty acid ethyl ester production during beer fermentation through *EEB1* and *ETR1* overexpression in *Saccharomyces pastorianus*. *Journal of Agricultural and Food Chemistry* **67**: 5607-5613.

Yoshida S, Imoto J, Minato T, Oouchi R, Sugihara M, Imai T, Ishiguro T, Mizutani S, Tomita M, Soga T & Yoshimoto H (2008) Development of bottom-fermenting *Saccharomyces* strains that produce high SO_2 levels, using integrated metabolome and transcriptome analysis. *Applied and Environmental Microbiology* 74: 2787-2796.

Yoshimoto H, Fujiwara D, Momma T, Ito C, Sone H, Kaneko Y & Tamai Y (1998) Characterization of the *ATF1* and *Lg-ATF1* genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*. *Journal of Fermentation and Bioengineering* **86**: 15-20.

Yoshimoto H, Fujiwara D, Momma T, Tanaka K, Sone H, Nagasawa N & Tamai Y (1999) Isolation and characterization of the *ATF2* gene encoding alcohol acetyltransferase II in the bottom fermenting yeast *Saccharomyces pastorianus*. *Yeast* **15**: 409-417.

Yu J, Vasanthan T & Temelli F (2001) Analysis of phenolic acids in barley by High-Performance Liquid Chromatography. *Journal of Agricultural and Food Chemistry* **49**: 4352-4358.

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV & Zhang F (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**: 759-771.

Zhang Y, Wang J, Wang Z, Zhang Y, Shi S, Nielsen J & Liu Z (2019) A gRNA-tRNA array for CRISPR-Cas9 based rapid multiplexed genome editing in *Saccharomyces cerevisiae*. *Nature Communications* **10**: 1053.

Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, Lan N, Jansen R, Bidlingmaier S, Houfek T, Mitchell T, Miller P, Dean RA, Gerstein M & Snyder M (2001) Global analysis of protein activities using proteome chips. *Science* **293**: 2101-2105.

Chapter 2

gEL DNA, a cloning- and PCR-free method for CRISPR-based multiplexed genome editing

Paola Randazzo, Nicole X. Bennis, Jean-Marc G. Daran & Pascale Daran-Lapujade

This chapter has been published in The CRISPR Journal (2021)

Abstract

Even for the genetically accessible yeast *Saccharomyces cerevisiae*, the CRISPR-Cas DNA editing technology has strongly accelerated and facilitated strain construction. Several methods have been validated for fast and highly efficient single editing events and diverse approaches for multiplex genome editing have been described in literature by means of *Spy*Cas9 or *Fn*Cas12a endonucleases and their associated gRNAs. The gRNAs used to guide the Cas endonuclease to the editing site are typically expressed from plasmids using native PolII or PolIII RNA polymerases. These gRNA-expression plasmids require laborious, time-consuming cloning steps, which hampers their implementation for academic and applied purposes. In this study, we explore the potential of expressing gRNA from linear DNA fragments using the T7 RNA polymerase (T7RNAP) for single and multiplex genome editing in *S. cerevisiae*. Using *Fn*Cas12a, this work demonstrates that transforming short, linear DNA fragments encoding gRNAs in yeast strains expressing T7RNAP, promotes highly efficient single and duplex DNA editing. These DNA fragments can be custom-ordered, which makes this approach highly suitable for high-throughput strain construction. This work expands the CRISPR-toolbox for large-scale strain construction programs in *S. cerevisiae* and promises to be relevant for other, less genetically accessible yeast species.

2.1. Introduction

The bacterial-derivative CRISPR-Cas technology is nowadays the most commonly used tool for microbial genome engineering. For the eukaryotic model and industrial workhorse *Saccharomyces cerevisiae*, several CRISPR-based methodologies have been developed, aiming at a fast and efficient single editing event (DiCarlo *et al.*, 2013, Gao & Zhao, 2014, Bao *et al.*, 2015, Mans *et al.*, 2015). Two Class II bacterial endonucleases, Cas9 and Cas12a (also known as Cpf1) have been functionally characterized for DNA editing, ranging from point mutation to heterologous pathway integration (Mans *et al.*, 2015, Swiat *et al.*, 2017, Verwaal *et al.*, 2018). While diverse Cas9- and Cas12a-mediated approaches for multiplex genome editing have been described in literature (reviewed

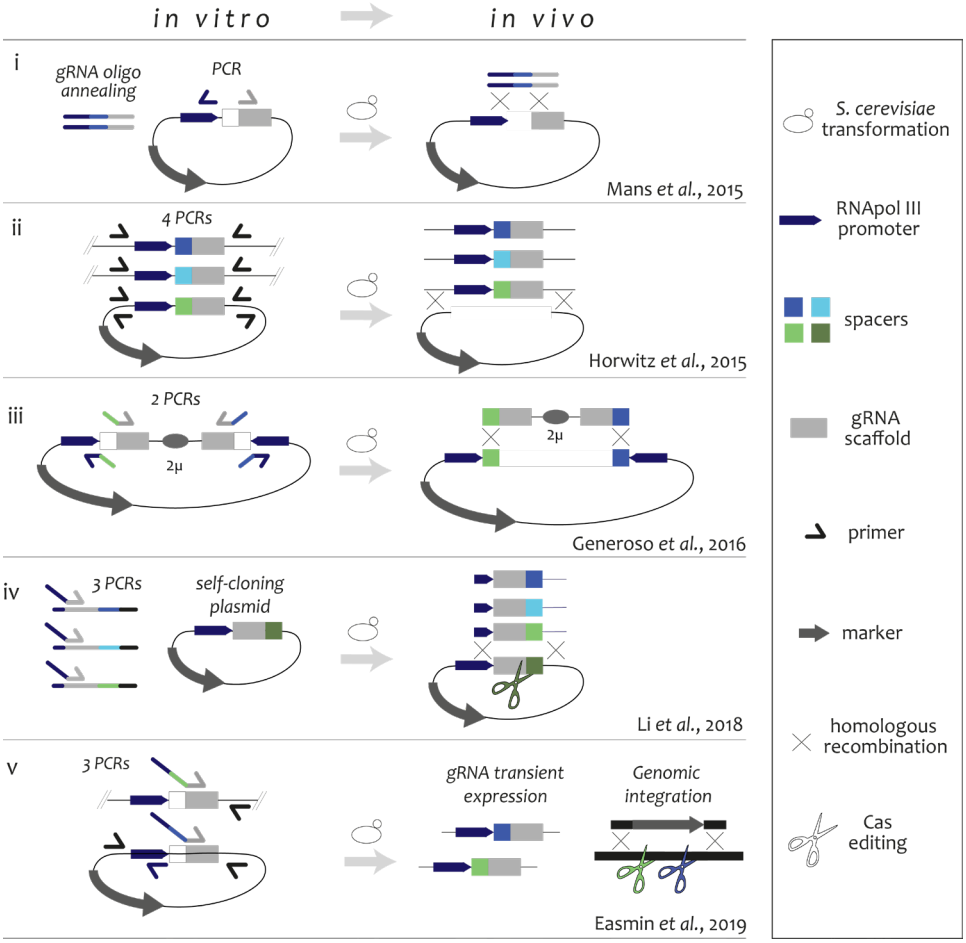


Figure 2.1. Cloning-free approaches for CRISPR-Cas-aided DNA editing. Overview of methodologies based on delivery of linear DNA templates for gRNAs expression in *Saccharomyces cerevisiae*. *In vitro* sample preparations and *in vivo* events upon transformation are described. All gRNA expression cassettes include an RNA polymerase III (RNAPolIII) terminator. Number of polymerase chain reactions (PCRs) are quoted. Features are depicted in the legend on the right-hand side of the figure.

in Adiego-Pérez *et al.*, 2019), multiplex genome editing still requires substantial efforts for the CRISPR tools to be built. The RNA molecules designed to guide the endonuclease towards the editing site (gRNAs) are typically cloned in and expressed from plasmids. In most published works so far, multiplex editing relies on the parallel transformation of multiple plasmids carrying a single or two gRNAs. However, this approach is limited by the number of available marker-based plasmid backbones (Ryan *et al.*, 2014, Horwitz *et al.*, 2015, Jakočiūnas *et al.*, 2015, Mans *et al.*, 2015, Walter *et al.*, 2016, Li *et al.*, 2018). More recently, several successful examples have shown that multiple gRNAs can be expressed from a single gRNA-array, using different tricks to release the mature gRNAs (Ryan & Cate, 2014, Bao *et al.*, 2015, Generoso *et al.*, 2016, Swiat *et al.*, 2017, Ferreira *et al.*, 2018, Li *et al.*, 2018, Verwaal *et al.*, 2018, Zhang *et al.*, 2019). However, these gRNA expression cassettes with their tailored sequence design and sequence complexity may be difficult to synthesize and require laborious and time-consuming cloning steps, therefore hindering the workflow for strain construction. To date, few attempts have been developed to circumvent gRNA cloning for genome editing of microbes in general and of *S. cerevisiae* in particular (illustrated in Figure 2.1).

The most straightforward, cloning-free strategy would rely on the delivery of the gRNA in the form of a short, linear DNA fragment. Such short DNA fragments could easily be synthetized as oligonucleotides and delivered as mixture in any desired gRNA combination for multiplex targeting of DNA sites. Such a cost-effective and versatile approach would be highly suited for high-throughput, multiplex genome engineering of strains. Transient expression of linear DNA carrying gRNA expression cassettes has been previously shown to enable Cas9-mediated DNA-editing (Horwitz *et al.*, 2015, Easmin *et al.*, 2019). However, these approaches systematically require a first in vitro step for the construction of vectors from which the linear DNA is produced by PCR amplification (Figure 2.1). In eukaryotes, gRNAs are transcribed either by RNA polymerase III (RNApolIII) or by RNA Polymerase II (RNAPolII), and have to be flanked by self-processing ribozymes or tRNAs that prevent unwanted processing of the gRNAs (Ryan & Cate, 2014, Zhang *et al.*, 2019). A recent report has shown that functional gRNAs can also be transcribed in different yeasts by the RNA polymerase from bacteriophage T7 (T7RNAP) localized in the nucleus (Morse *et al.*, 2018). Delivered as plasmid DNA, the T7RNAP-transcribed gRNAs have been used to guide Cas9 for genome editing and dCas9 for transcriptional regulation.

The present work introduces the gEL DNA method, a novel, utterly cloning and PCR-free genome editing tool, based on the **g**RNA **E**xpression from short, **L**inear double-stranded **DNA** oligos by the T7RNAP (Figure 2.2). Comparing *Spy*Cas9 and *Fn*Cas12a, this study demonstrates that *Fn*Cas12a enables efficient single and multiplexed DNA editing from custom-ordered oligonucleotides of 87 nt in *S. cerevisiae*. Next to gRNA *in silico* design, the only steps required for genome editing are transformation and screening. Highly suited for high-throughput strain construction, the gEL DNA method

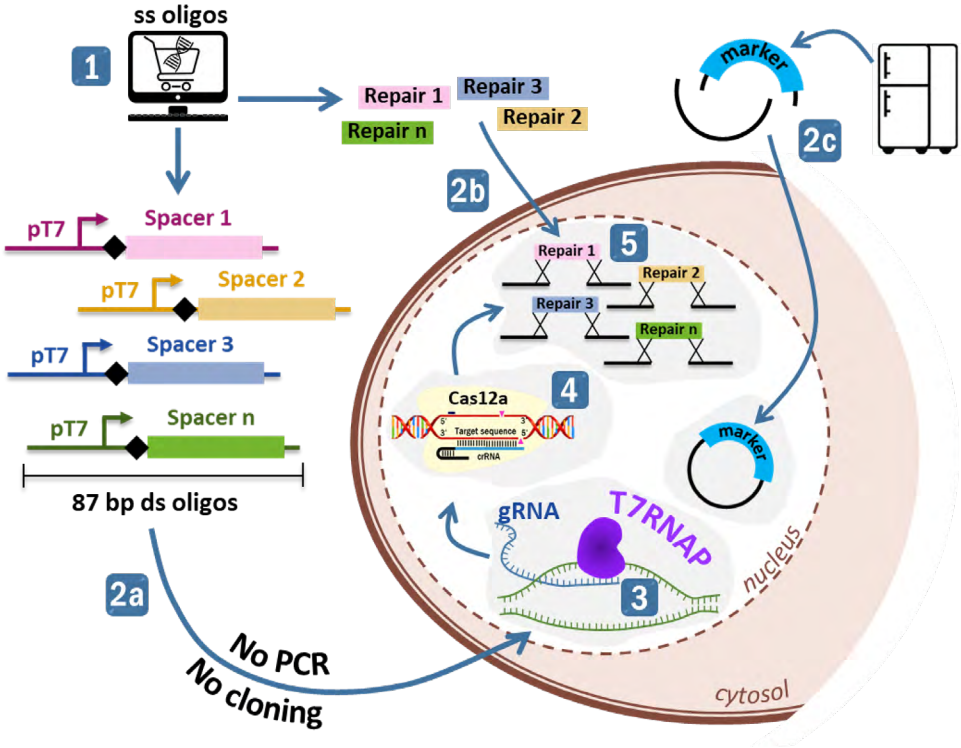


Figure 2.2. Schematic overview of the gEL DNA approach: **1**, *in silico* design and ordering of gDNA cassettes (87 bp) and repair DNA (120 bp) as oligos; **2**, transformation with the double-stranded (ds) gDNA expression cassettes **(2A)**, the ds repair DNA fragments **(2B)**, and an empty, split plasmid carrying a marker of choice **(2C)**; **3**, expression of the gRNA by the T7RNAP; **4**, targeted DNA editing by *Fn*Cas12a; **5**, repair of the dsDNA break via homologous recombination using the repair DNA fragments.

does not require prior knowledge on the transcription machinery of the host microbe (e.g. RNA processing and promoters) and thereby promises to facilitate DNA editing in less genetically accessible microbes.

2.2. Materials and Methods

Strains and cultivation conditions

All *S. cerevisiae* strains used in this study (Table 2.1) were derived from the CEN.PK background strain (Entian & Kotter, 2007). Yeast cells were grown at 30 °C in shake flasks on rotary shaker (200 rpm) or on agar plates (20 g L^{-1}). Complex medium contained 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose (YPD). YPD was supplemented with nourseothricin (100 mg L^{-1}), geneticin (G418) (200 mg L^{-1}) or hygromycin B (200 mg L^{-1}) to select transformants. Minimal synthetic media were prepared as previously described (Verduyn *et al.*, 1992). SMD medium contained 5 g L^{-1} $(NH_4)_2SO_4$, 3 g L^{-1}

Table 2.1. *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Origin
CEN.PK113-7D	*MATa MAL2–8c SUC2*	(Entian & Kotter, 2007)
IMX1714	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2*	This study
IMX1752	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a*	This study
IMX1905	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a* YPRCτ3Δ::T7RNAP^{K276R}	This study
IMX2030	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a* YPRCτ3Δ::T7RNAP^{P266L,K276R}	This study
IMX2031	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a* YPRCτ3Δ::T7RNAP	This study
IMX2032	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a* YPRCτ3Δ::T7RNAP^{P266L}	This study
IME459	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a* pUDE866	This study
IME460	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a* pGGKd034	This study
IME475	*MATa MAL2–8c SUC2 sga1Δ::Spycas9-natNT2, X-2(*)::Fncas12a pUDE911*	This study
IME638	*MATa MAL2–8c SUC2* pUDE911	This study
IME639	*MATa MAL2–8c SUC2* pUDE1082	This study
IME640	*MATa MAL2–8c SUC2* pUDE1083	This study
IME641	*MATa MAL2–8c SUC2* pUDE1084	This study
IME642	*MATa MAL2–8c SUC2* pGGKd034	This study
IME645	*MATa MAL2–8c SUC2* pUDE1086	This study
IME646	*MATa MAL2–8c SUC2* pUDE1087	This study

(*) Integration site at 194944-195980 of Chromosome X from Mikkelsen *et al.* (2012)

KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 ml L^{-1} trace element solution, supplemented with 20 g L^{-1} of glucose and 1 ml L^{-1} vitamin solution. SMD-urea included 6.6 g L^{-1} K_2SO_4, 3.0 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mL L^{-1} trace element solution, supplemented with 20 g L^{-1} of glucose, 1 ml L^{-1} of a vitamin solution and 2.3 g L^{-1} CH_4N_2O (Milne *et al.*, 2015). Utilization of urea as nitrogen source instead of ammonium prevents excessive acidification of the medium resulting from ammonium uptake, and enables the culture pH to be maintained close to the initialy set value. For selection of transformants carrying the *amdS* marker cassette, ammonium sulfate in SMD was substituted with 10 mM acetamide and 6.6 g L^{-1} K_2SO_4 (SM-Ac) (Solis-Escalante *et al.*, 2013). Plasmids were propagated in *Escherichia coli* XL1-Blue cells (Agilent Technologies, Santa Clara, CA), by growing the cells in liquid Lysogeny broth (LB) medium composed of 10 g L^{-1} tryptone,

5 g L^{-1} yeast extract and 10 g L^{-1} NaCl, supplemented with chloramphenicol (25 mg L^{-1}), spectinomycin (100 mg L^{-1}) or ampicillin (100 mg L^{-1}) at 37 °C. Solid LB medium was obtained by addition of 20 g L^{-1} agar. When required, plasmids from yeasts isolates were removed accordingly to described procedures (Mans *et al.*, 2015). All *S. cerevisiae* and *E. coli* stocks were prepared by aseptically adding 30% v/v of glycerol to exponentially growing cultures. Aliquoted cell stocks were stored at -80 °C.

Molecular biology techniques

Yeast genomic DNA used for cloning purposes was isolated using the YeaStar genomic DNA kit (Zymo Research, Irvine, CA) according to manufacturer's instructions. Diagnostic PCR was performed using DreamTaq DNA polymerase (Thermo Fisher Scientific, Walthman, MA). For cloning and sequencing purposes, PCR products were obtained using Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Primers were ordered as PAGE or desalted purified oligonucleotides (Table S1) from Sigma-Aldrich (St Louis, MO). Annealed oligos were quantified by BR dsDNA kit using the Qubit spectrophotometer (Invitrogen, Carlsbad, CA). DNA fragments were separated by electrophoresis on 1% (w/v) or 2% (w/v) agarose gels, depending on the fragment size. PCR products were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), after restriction digestion of the PCR mixture with DpnI (Thermo Fisher Scientific) for removal of templates. When required, DNA fragments were excised from gel and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Plasmids were isolated from *E. coli* cultures using the Sigma GenElute™ Plasmid Miniprep kit (Sigma-Aldrich).

Entry-vector plasmids construction

All plasmids used in this study are listed in Table 2.2. The pUD565 plasmid (Hassing *et al.*, 2019), a GFP dropout (GFPdo) entry vector compatible with Yeast Toolkit parts (Lee *et al.*, 2015), was ordered as synthetic gene from GeneArt (Thermo Fisher Scientific). GFPdo entry vectors for cloning of transcriptional units were constructed following the BsaI Golden Gate reaction protocol described by Lee *et al.* (2015). The GFPdo pGGKd018 plasmid was obtained by assembly of part plasmids pYTK002, pYTK047, pYTK067, pYTK077, pYTK082 and pYTK085. The GFPdo pGGKd034 plasmid was constructed by assembly of part plasmids pYTK002, pYTK047, pYTK067, pYTK079, pYTK082 and pYTK083. The GFPdo pUDE810, an entry vector for exprssion of *Fn*Cas12a crRNAs, was constructed by Golden Gate assembly of pre-annealed primers 12647 & 12648 with the following PCR-generated fragments: the pGGKd018 backbone with primers 12799-12800; the *SNR52* promoter amplified from the pMEL13 template (Mans *et al.*, 2015) using primers 12645 & 13546; the GFPdo cassette bearing specific BsaI overhangs (GATC and ATCC) obtained by PCR amplification with primers 13547 & 12644 on pYTK047 (Lee *et al.*, 2015).

Table 2.2. List of plasmids used in this study

Plasmid	Genotype[a]	Reference
pUG-natNT2	*ampR natMX*	(de Kok *et al.*, 2012), Addgene #110922
pYTK002	*camR ConLS*	(Lee *et al.*, 2015), Addgene #65109
pYTK013	*camR pTEF1*	(Lee *et al.*, 2015), Addgene #65120
pYTK027	*camR pREV1*	(Lee *et al.*, 2015), Addgene #65134
pYTK036	*camR Spycas9*	(Lee *et al.*, 2015), Addgene #65143
pYTK047	*camR GFPdo*	(Lee *et al.*, 2015), Addgene #65154
pYTK051	*camR tENO1*	(Lee et al., 2015), Addgene #65158
pYTK067	*camR ConR1*	(Lee *et al.*, 2015), Addgene #65174
pYTK077	*camR KanMX*	(Lee *et al.*, 2015), Addgene #65184
pYTK079	*camR HygR*	(Lee *et al.*, 2015), Addgene #65186
pYTK082	*camR 2 µm*	(Lee *et al.*, 2015), Addgene #65189
pYTK083	*ampR ColE1*	(Lee *et al.*, 2015), Addgene #65190
pYTK085	*specR ColE1*	(Lee *et al.*, 2015), Addgene #65192
pUDE483	*2 µm ampR pTEF1p-Spycas9-tENO1*	This study
pUDC175	*CEN6/ARS4 ampR TRP1 pTEF1-Fncas12a-tCYC1*	(Swiat *et al.*, 2017), Addgene #103019
pUDR573	*2 µm ampR amdS* sgRNA X-2	(Baldi *et al.*, 2019)
pRS315-nls-T7-RNAP	*CEN6/ARS4 ampR LEU2 pTDH3*-T7RNAP^{K276R}-*tTDH3*	(Dower & Rosbash, 2002), Addgene #33152
pUD565	*camR* GFPdo part entry vector	GeneArt
pGGKp172	*camR* T7RNAP^{K276R}	This study
pGGKp035	*camR pTDH3*	(Hassing *et al.*, 2019)
pGGKp039	*camR tTEF1*	(Hassing *et al.*, 2019)
pGGKp100	*camR pPFK1*	(Boonekamp *et al.*, 2018)
pGGKd034	*2 µm ampR HygR GFPdo*	This study
pUDE866	*2 µm ampR HygR pTDH3*-T7RNAP^{K276R}-*tTEF1*	This study
pUDR477	*2 µm specR KanMX crYPRτ3.3*	This study
pGGKd018	*2 µm specR KanMX* GFPdo	This study

Table 2.2. (continued)

Plasmid	Genotype[a]	Reference
pMEL13	2 µm *ampR KanMX* sgRNA *CAN1.Y*	(Mans *et al.*, 2015), Euroscarf P30782
pUDE810	2 µm *specR KanMX pSNR52-GFPdo-tSUP4*	This study
pUDE759	2 µm *specR KanMX pSNR52-crADE2-3s-tSUP4*	This study
pUDR482	2 µm *specR KanMX pSNR52-G-crADE2-3s-tSUP4*	This study
pUDR483	2 µm *specR KanMX pSNR52-GG-crADE2-3s-tSUP4*	This study
pUDR484	2 µm *specR KanMX pSNR52-GGG-crADE2-3s-tSUP4*	This study
pUDR485	2 µm *specR KanMX S.T7p-crADE2-3s-T7t*	This study
pUDR486	2 µm *specR KanMX S.T7p-G-crADE2-3s-T7t*	This study
pUDR487	2 µm *specR KanMX S.T7p-GG-crADE2-3s-T7t*	This study
pUDR488	2 µm *specR KanMX S.T7p-GGG-crADE2-3s-T7t*	This study
pUDR489	2 µm *specR KanMX L.T7p-crADE2-3s-T7t*	This study
pUDR490	2 µm *specR KanMX L.T7p-G-crADE2-3s-T7t*	This study
pUDR491	2 µm *specR KanMX L.T7p-GG-crADE2-3s-T7t*	This study
pUDR492	2 µm *specR KanMX L.T7p-GGG-crADE2-3s-T7t*	This study
pUDR585	2 µm *specR KanMX pSNR52-GGG-sgRNA-ADE2.Y-tSUP4*	This study
pUDR579	2 µm *specR KanMX S.T7p-GGG-sgRNA-ADE2.Y-T7t*	This study
pUDR581	2 µm *specR KanMX L.T7p-GGG-sgRNA-ADE2.Y-T7t*	This study
pUDR506	2 µm *ampR KanMX* gRNA T7RNAP	This study
pUDE911	2 µm *ampR HygR pTDH3*-T7RNAP^{P266L}-*tTEF1*	This study
pUDE710	2 µm *KanMX ampR pSNR52-crADE2-3.crHIS4–4-tSUP4*	(Swiat *et al.*, 2017), Addgene #103020
pUDE735	2 µm *KanMX ampR pSNR52-crCAN1–4.crHIS4–4. crPDR12–3.crADE2–3-tSUP4*	(Swiat *et al.*, 2017), Addgene #103024
pUDR692	2 µm *KanMX ampR pSNR52-crCAN1–3.crHIS4–4. crPDR12–3.crADE2–3-tSUP4*	This study
pUDR715	2 µm *specR KanMX pSNR52-GGG-crHIS4-4s-tSUP4*	This study
pUDR716	2 µm *specR KanMX pSNR52-GGG-crPDR12-3s-tSUP4*	This study
pUDR717	2 µm *specR KanMX pSNR52-GGG-crCAN1-4s-tSUP4*	This study
pUDR718	2 µm *specR KanMX pSNR52-GGG-crCAN1-3s-tSUP4*	This study
pUDE1082	2 µm *ampR HygR pPFK1-Fncas12a-tCYC1*	This study
pUDE1083	2 µm *ampR HygR pPFK1-Fncas12a-tCYC1 pTDH3*-T7RNAP^{P266L}-*tTEF1*	This study
pUDE1084	2 µm *ampR HygR pTEF1-Fncas12a-tCYC1 pTDH3*-T7RNAP^{P266L}-*tTEF1*	This study
pUDE1086	2 µm *ampR HygR pREV1-Fncas12a-tCYC1*	This study
pUDE1087	2 µm *ampR HygR pREV1-Fncas12a-tCYC1 pTDH3*-T7RNAP^{P266L}-*tTEF1*	This study

[a] 'sgRNA' denotes single-guide RNA used by Cas9, 'cr' refers to crRNA for *Fn*Cas12a. The presence of an 's' following the crRNA indicates that a shorter spacer of 19 nt is used, otherwise the spacer size is 25 nt.

Construction of the dual Cas-expressing strain IMX1752

The construct for genomic integration of *Spy*Cas9 gene consisted of a paired expression cassette for introduction of *Streptococcus pyogenes Spycas9* nuclease and *natNT2* marker into the *SGA1* locus (Mans *et al.*, 2015). First, the *natNT2* marker was PCR-amplified from pUG-natNT2 (Addgene plasmid #110922 (de Kok *et al.*, 2012)) using primers 10297 & 10298. This PCR product was cloned via Golden Gate together with pre-annealed primer pairs 10293 & 10294 and 10295 & 10296, and Yeast toolkit plasmids pYTK013, pYTK036, pYTK051, pYTK082 and pYTK083 (Lee *et al.*, 2015), resulting in plasmid pUDE483. The *Spycas9-natNT2* integration cassette was obtained by enzyme restriction of pUDE483 using EcoRI. The restriction mix was directly transformed into *S. cerevisiae* using the lithium acetate (LiAc) transformation protocol (Gietz & Schiestl, 2007). Transformants were selected on YPD supplemented with nourseothricin. A single isolate, which was renamed IMX1714 (Table 2.1), was submitted to an additional transformation for the genomic integration of the *Fn*Cas12a nuclease. For this, the sequence of *Francisella novicida cas12a* was amplified from pUDC175 (Addgene plasmid #103019 (Swiat *et al.*, 2017)) using primers 13553 & 13554. The obtained PCR product, carrying 60 bp homology flanks to the X-2 integration site (Mikkelsen *et al.*, 2012), was transformed in IMX1714 as previously described in (Gietz & Schiestl, 2007), together with plasmid pUDR573 (Baldi *et al.*, 2019) for *Spy*Cas9-mediated targeting at this genomic site. Transformants were selected on SM-Ac plates. Correct genomic integrations were confirmed by diagnostic PCR using primers listed in Table S1. After removal of the gRNA expression plasmid, the dual *Spy*Cas9/*Fn*Cas12a *S. cerevisiae* strain was stocked as IMX1752.

Construction of the T7RNAP^{K276R}-expressing strain IMX1905

First, the T7RNAP^{K276R} sequence was PCR-amplified from plasmid pRS315-NLS-T7-RNAP (Addgene plasmid #33152) (Dower & Rosbash, 2002) using primers 13543 & 13544, and the obtained PCR fragment stably cloned into entry vector pUD565, resulting in part plasmid pGGKp172. The T7RNAP^{K276R} transcriptional unit was assembled by Golden Gate cloning into plasmid pGGKd034, together with part plasmids pGGKp035 (*pTDH3*) and pGGKp039 (*tTEF1*) (Hassing *et al.*, 2019), leading to plasmid pUDE866.

For genomic integration of the T7RNAP^{K276R}, the previously characterized YPRCτ3 site of the *S. cerevisiae* genome was chosen as recipient locus (Flagfeldt *et al.*, 2009). Thus, a gRNA for *Fn*Cas12a-mediated editing at this site was designed accordingly to guidelines provided in Swiat *et al.*, (2017). The gRNA for integration in YPRCτ3 was ordered as oligos 14142 & 14143 containing specific overhangs for Golden Gate assembly (GATC and ATCC). Oligo annealing and cloning into pUDE810 plasmid resulted in the crRNA-expressing plasmid pUDR477. Amplification of the T7RNAP^{K276R} integrative cassette was carried out on the pUDE866 plasmid using primers 14022 & 14023, which contain repair ends of 60 bp homologous to the YPRCτ3 locus. These generated PCR fragments

were co-transformed with plasmid pUDR477 into IMX1752 cells, as previously described (Gietz & Schiestl, 2007). Yeast cells were selected on solid YPD plates supplemented with G418. Diagnostic PCR was performed on a single colony isolate, plasmid was recycled and the constructed strain was renamed IMX1905.

Construction of T7RNAP mutants and T7RNAP-overexpressing strains

In order to alter the T7RNAP protein sequence, the T7RNAP gene of IMX1905 was *in vivo* mutated by means of the CRISPR-*Spy*Cas9 editing machinery. A single gRNA was chosen for targeting the sequence surrounding DNA encoding amino acids at positions 266 and 276 (corresponding to 276 and 286 if considering the nuclear localization signal (NLS)) (Figure S3). For this, oligo 14284 was Gibson-assembled by bridging to the pMEL13 (Mans *et al.*, 2015) backbone, which was previously PCR-amplified using primers 6005 & 6006. The obtained plasmid was renamed pUDR506. Repair oligos (Table S1) consist of 120 bp surrounding the T7RNAP target sequence with SNPs for P266L and/or R276K mutations, and carrying a silent mutation at the PAM sequence to avoid reiterative cutting. Plasmid pUDR506 and each of the double-stranded repair oligos were co-transformed into competent IMX1905 cells (Gietz & Schiestl, 2007). Transformants were plated on YPD agar supplemented with G418. Screening of eight selected colonies was performed by SNP genotyping with primers listed in Table S1, following previously described procedures for SNP scoring (Meijnen *et al.*, 2016). After SNPs validation and Sanger sequencing of the mutated T7RNAP sequence (Figure S3), strains were stocked as follows: IMX2030 (T7RNAP$^{P266L, K276R}$) was renamed after the P266L amino acid substitution; IMX2031 (T7RNAP^{WT}) expresses the wild-type T7RNAP, where the arginine at position 276 is changed into the native lysine; IMX2032 (T7RNAP^{P266L}) resulted from simultaneous mutations of proline and arginine at positions 266 and 277 for the respective amino acid change into leucine and lysine.

For T7RNAP^{K276R} overexpression, the dual Cas-expressing strain IMX1752 was transformed with plasmid pUDE866, following standard practice (Gietz & Schiestl, 2007). Transformants were selected on YPD plates supplemented with hygromycin B and the strain was renamed as IME459. In parallel, transformation of IMX1752 with the empty vector pGGKd034 led to the control strain IME460. To overexpress the T7RNAP^{P266L} variant, the gene sequence of strain IMX2032 was PCR-amplified from its isolated genomic DNA, using primers 10753 & 10768. The obtained PCR product was cloned by Golden Gate assembly into the episomal entry plasmid pGGKd034. The obtained plasmid, renamed pUDE911, was transformed into IMX1752, transformants plated on selective YPD hygromycin B medium and selected colonies were stocked as IME475.

Construction of the *Fncas12a*-T7RNAP^{K276R} transportable system

To construct plasmids carrying *Fncas12a* expression cassettes, two alternative

promoters were cloned by Golden Gate into the pGGKd034 vector together with the PCR product obtained by amplifying pUDC175 using primers 18075 & 18076. Plasmid pGGKp100 (*pPFK1*) (Boonekamp *et al.*, 2018) was used for assembly of pUDE1082, while plasmid pYTK027 (*pREV1*) (Lee *et al.*, 2015) was used for the construction of pUDE1086. Cloning of the transportable *Fncas12a*-T7RNAP^{K276R} system was performed by Gibson assembly using PCR amplicons with synthetic flags of 60 bp. Flagged primers 18077 & 18166 were used for PCR linearization of plasmid pUDE911. Flagged *Fncas12a* expression cassettes were amplified from pUDC175, pUDE1082 or pUDE1086 templates using primers 11868 & 10189, 18078 & 10189 or 18132 & 10189, respectively. Gibson assembly of these alternative *Fn*Cas12a expression cassettes into the linearized pUDE911 resulted in assembly of the respective plasmids pUDE1083, pUDE1084 and pUDE1087. CEN.PK113-7D transformed with each of the episomal plasmids were stocked as strains IME638 (pUDE911), IME639 (pUDE1082), IME640 (pUDE1083), IME641 (pUDE1084), IME642 (pGGKd034), IME645 (pUDE1086) and IME646 (pUDE1087). All transformants were selected on selective YPD hygromycin B plates.

Assembly of gRNA expression cassettes

The gRNA cassettes for evaluation of *ADE2* deletion efficiencies mediated by *Fn*Cas12a or *Spy*Cas9 nucleases were prepared using the highly-efficient *ADE2-3* (Swiat *et al.*, 2017) or the *ADE2.y* (DiCarlo *et al.*, 2013) gRNAs, respectively. Each gRNA cassette was expressed from high-copy plasmid and comprised the gRNA sequence left-flanked by the RNAPolIII-dependent *SNR52p*, the minimal T7p 'TAATACGACTCACTATA' (S.T7p) or an extended T7p 'GCCGGGAATTTAATACGACTCACTATA' (L.T7p), with respective terminator sequences at the right flank. For the *Fn*Cas12a-mediated targeting of other genes, previously characterized gRNAs were expressed from a single gRNA-expressing cassette or from a gRNA array: *HIS4* (*HIS4-4*), *PDR12* (*PDR12-3*) or *CAN1* (*CAN1-4* or *CAN1-3*) (Swiat *et al.*, 2017).

All single gRNA-expressing plasmids were assembled by Gibson assembly reaction using the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs). Depending on the plasmid features, the backbone of the pUDE810 plasmid was amplified using different primer couples for specific homology overhangs. The backbone for assembly of *Fn*Cas12a-gRNAs with *pSNR52/tSUP4* flanks was obtained by PCR amplification with primers 12710 & 5793. For T7RNAP-mediated expression of gRNAs for *Fn*Cas12a, plasmid backbone was obtained by PCR amplification using either primers 14274 & 13713 (S.T7p/T7t) or 14275 & 13713 (L.T7p/T7t). Plasmid pUDE759 was constructed by assembly of the *pSNR52p/tSUP4* backbone fragment with annealed oligos 12713 & 12714. Single oligos were used for the Gibson assembly of *Fn*Cas12a-gRNAs cassettes by single-stranded DNA bridging to each individual PCR-originated backbone fragments: the *pSNR52/tSUP4* derivatives pUDR482 (primer 14282), pUDR483 (primer 13750), pUDR484 (primer 14283), pUDR715 (primer 17328), pUDR716 (primer 17329), pUDR717

(primer 17330), pUDR718 (primer 17331); the S.T7p/T7t-related plasmids pUDR485 (primer 14280), pUDR486 (primer 13751), pUDR487 (primer 14281), pUDR488 (primer 13572) and the L.T7p/T7t cognate plasmids pUDR489 (primer 14276), pUDR490 (primer 14277), pUDR491 (primer 14278) and pUDR492 (primer 14279).

For assembly of *Spy*Cas9-gRNAs under *pSNR52*, the amplified pUDE810 backbone with *pSNR52/tSUP4* edges was mixed with annealed oligos 15508 & 15509 and the single-stranded oligo 14426 in a Gibson reaction, resulting in plasmid pUDR585. For *Spy*Cas9-gRNAs expressed by T7RNAP, T7-edged plasmid backbones were PCR amplified using primers 14274 & 15287 (S.T7p/sgRNA-T7t) or 14275 & 15287 (L.T7p/sgRNA-T7t), generating a dsDNA fragment that additionally contains a partial sequence of the gRNA scaffold for *Spy*Cas9. Gibson assemblies of annealed oligos 15290 & 15291 to either the S.T7p/sgRNA-T7t or S.T7p/sgRNA-T7t PCR-generated backbones were performed to obtain plasmids pUDR579 and pUDR581, respectively.

The control array of gRNAs expressed by plasmid pUDR692 was ordered as synthetic gene from GeneArt (Thermo Fisher Scientific). *SNR52* promoter and gRNA design principles previously elucidated were used (Swiat *et al.*, 2017). The synthetic gRNA array was flanked by BsaI sites and assembled by Golden Gate cloning into pGGKd018.

Delivery methods of gRNA expression cassettes

Each gRNA expression cassette was transformed together with 1 µg of double-stranded deletion repair (Table S1) in exponentially growing *S. cerevisiae* cells (~2*10^7 cells ml^{-1}), accordingly to the LiAc transformation protocol (Gietz & Schiestl, 2007). Genome editing via *in vitro* assembly described in Table 2.3 was performed by transforming 500 ng (~150 fmol) of each gRNA expression plasmid. For genome editing achieved via *in vivo* plasmid assembly, two linear PCR fragments were delivered with the transformation mix: i) 150 fmol of the specific gRNA cassette, systematically amplified from the respective *in vitro* constructed plasmid using primers 14584 & 14585; ii) 150 fmol of the linearized marked 2µ backbone with 60 bp homology to each gRNA cassette, obtained from the amplification of pUDE810 with primers 11571 & 12378. To evaluate genome editing via delivery of a linear gRNA expression cassette, each amplified gRNA cassette (150 fmol) was transformed with either 500 ng of circular pGGKd018 plasmid or with equimolar amount of two PCR fragments for the split plasmid selection using pGGKd018. These amplicons, having homologies for *in vivo* recircularization of the plasmid, were obtained by PCR amplification with primers 6815 & 9340 and primers 2398 & 12097.

All transformations were plated on selective YPD medium supplemented with G418. Efficiency of *ADE2* deletion is measured as number of red colonies on total colony forming units (CFU). For editing of other sites, diagnostic PCR was performed on a number of selected colonies using primers listed in Table S1.

Preparation of gDNAs and genome editing via gEL DNA

Sequences of deletion repair fragments and gDNAs are listed as forward and reverse oligonucleotides in Table S1. Site XI-3 is located at the previously characterized integration locus on chromosome XI (Mikkelsen *et al.*, 2012), while XVI-1 corresponds to the intergenic region between *CUP9* and *TRE1* on chromosome XVI. The spacer for the non-targeting (nt) gDNA was designed by scrambling the *ADE2-3s* spacer sequence. Each forward and reverse oligo was mixed in equimolar amount, heated for 5 minutes at 95°C and cooled down to room temperature. As only exception, *Spy*Cas9-mediated editing using gDNA with long T7 promoter was performed using PCR amplification of two overlapping primers, the gRNA-specific forward for *ADE2.y* (16745) and the universal reverse carrying the *Spy*Cas9-gRNA scaffold (16746). Concentrations of each double-stranded annealed oligos were measured for all pre-annealed oligos or the non-purified PCR-derived gDNA. 1 µg of each deletion repair and 4 µg of respective gDNA were mixed with 500 ng of split pGGKd18 plasmid for selection purposes and transformed into competent T7RNAP-expressing yeast cells accordingly to standard procedure (Gietz & Schiestl, 2007). Transformants were selected on YPD plates supplemented with G418 when transforming strains with genomically integration T7RNAP (IMX1905, IMX2030, IMX2031, IMX2032), or with G418 and hygromycin B when transforming strains that overexpress T7RNAP from a plasmid (IME459, IME475) or for selection of the dual *Fncas12a*-T7RNAP overexpression strain (IME641). Plasmid-based controls for multiplex editing via *Fn*Cas12a gRNA-arrays were performed according to Swiat *et al.*, 2017. Diagnostic PCR of selected colonies was done using primers listed in Table S1.

Growth rate measurement

Strains were cultivated in 96-well plates containing SMD medium or SMD-urea supplemented with hygromycin B (30°C, 250 rpm). Growth was monitored by measuring optical density at 660 nm at regular time intervals using the Growth Profiler 960 (Enzyscreen B.V., Heemstede, The Netherlands). Maximum specific growth rates (μ_{max}) were calculated using the equation: $X=X_0 e^{\mu t}$ in which µ indicates the exponential growth rate, from four independent biological cultures.

Bioinformatic analysis

The short sequence of the T7 promoter was mapped to the CEN.PK113-7D reference genome (Salazar *et al.*, 2017) using the Bowtie aligner (version 1.2.1.1) (Langmead *et al.*, 2009), with "--all" for reporting all alignments per input query. The RNA secondary structure was predicted with the RNAstructure Web Server (https://rna.urmc.rochester.edu/RNAstructureWeb/) (Bellaousov *et al.*, 2013). Temperature was set to 30°C (303.15 K). Self-folding free energies are obtained via the same webtool.

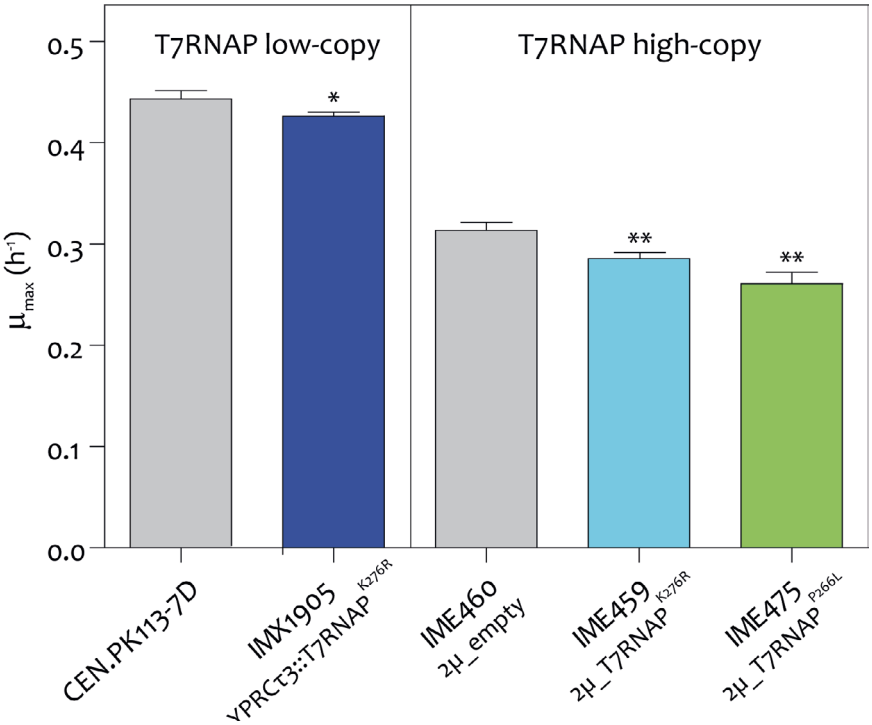


Figure 2.3. Physiological characterization of *S. cerevisiae* strains expressing T7RNAP. Maximum specific growth rates (μ_{max}) of *S. cerevisiae* constitutively expressing T7RNAP^{K276R}, *Spycas9*, and *Fncas12a* (IMX1905) and the control strain (CENPK.113-7D), or *S. cerevisiae* strains overexpressing T7RNAP^{K276R} (IME459) or T7RNAP^{P266L} (IME475) and the control strain carrying a 2μm multi-copy empty vector (IME460). All strains were cultivated on a 96-well plate containing chemically defined medium supplemented with glucose as the sole carbon source (SMD for CENPK.113-7D or IMX1905; SMD-urea with hygromycin B for IME459, IME475, or IME460; the slower growth rate measured for strains with plasmids compared to strains with genomic integration is explained by the difference in medium composition). Data points represent average and mean deviations of four biological replicates. *p < 0.025; **p < 0.001. Student's t-test was calculated compared to respective control strains CENPK.113-7D or IME460.

2.3. Results

T7RNAP-expressing *S. cerevisiae* as a platform strain for Cas-mediated genome editing

For T7 RNA polymerase (T7RNAP)-based expression of gRNAs, the bacteriophage T7RNAP, which was previously functionally expressed in the yeast nucleus, was chosen (Dower & Rosbash, 2002). Flanked by the strong and constitutive *TDH3* promoter and *TEF1* terminator, T7RNAP was integrated in the genome of a *S. cerevisiae* strain from the CEN.PK family that constitutively expressed both *Spycas9* and *Fncas12a* (strain IMX1752; Table 2.1). Sanger sequencing of the resulting strain IMX1905 revealed a missense mutation in the coding sequence of the T7RNAP as compared to the canonical sequence (https://www.uniprot.org/uniprot/P00573), which resulted in the replacement of a lysine by an arginine at the amino acid 276 of the polymerase

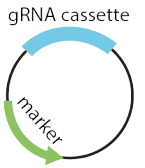
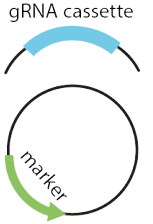
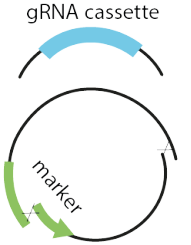
(corresponding to amino acid 286 when considering the NLS) (Figure S1). As the strain characterized by Dower and Rosbash, 2002 contained the same amino acid substitution and was proven to be functional in yeast, we decided to keep this variant (from now on referred to as T7RNAP^{K276R}) to test the gEL DNA approach.

Physiological characterization revealed that IMX1905, co-expressing T7RNAP^{K276R}, *Spycas9* and *Fncas12a*, grew as fast as the prototrophic control strain CEN.PK113-7D in chemically defined medium supplemented with glucose as sole carbon source (specific growth rate of 0.42 ± 0.01 h^{-1} for IMX1905 and 0.44 ± 0.01 h^{-1} for CEN.PK113-7D) (Figure 2.3). Expression of T7RNAP is therefore not toxic for *S. cerevisiae*.

T7RNAP enables gRNA expression from linear and circular DNA and promotes *Spy*Cas9- and *Fn*Cas12a-mediated DNA editing in *S. cerevisiae*

The activity of the T7RNAP^{K276R} in *S. cerevisiae* was evaluated by measuring the DNA editing efficiency of *Spy*Cas9 and *Fn*Cas12a guided by gRNAs expressed from T7 promoter. Two different T7 promoter lengths were tested, the minimal T7 promoter of 17 bp (TAATACGACTCACTATA; referred to as S.T7p), and an extended T7 promoter sequence of 27 bp (GCCGGGAATTTAATACGACTCACTATA; referred to as L.T7p) known to improve the stability of the T7RNAP-promoter complex *in vitro* (Tang *et al.*, 2005). An in-depth alignment search showed that the S.T7p sequence does not occur in the *S. cerevisiae* genome, making unwanted transcription from the host genome highly unlikely. T7RNAP^{K276R}-driven gRNA expression was compared with gRNA expression from the RNAPolIII-dependent *SNR52* promoter, largely adopted by the yeast community for CRISPR-Cas editing (DiCarlo *et al.*, 2013, Swiat *et al.*, 2017). Downstream all three promoters, at the initially transcribed region (ITS), a guanine triplet was added to increase the T7RNAP transcriptional activity (Imburgio *et al.*, 2000). As previously reported for *Spy*Cas9 (Morse *et al.*, 2018), the addition of this guanine triplet strongly improved T7RNAP-mediated expression for *Fn*Cas12a-based DNA editing (Figure S2). Disruption of *ADE2*, leading to a red colony phenotype, was used to assess editing efficiency (Dorfman, 1969) in strain IMX1905 (Table 2.1). Spacers previously shown to guide *Spy*Cas9 and *Fn*Cas12a to *ADE2* with high efficiency were chosen (DiCarlo *et al.*, 2013, Swiat *et al.*, 2017). *Spy*Cas9 and *Fn*Cas12a have different requirements for functionality, which results in different compositions and sizes of the DNA cassette encoding the gRNA (from now on called gRNA cassette) (Table 2.3). The *ADE2.y* gRNA for *Spy*Cas9 was used in its standard chimeric form including the trans-acting RNA (tracrRNA) (DiCarlo *et al.*, 2013). The *ADE2-3* gRNA for *Fn*Cas12a was reduced to the minimal 19 nt-long spacer enclosed by the matured direct repeats (DR), as recently described (Creutzburg *et al.*, 2020). Thanks to *Fn*Cas12a minimal requirements for DNA targeting and editing (no tracrRNA, small DR and spacer), the gRNA cassettes for *Fn*Cas12a were substantially smaller than those for *Spy*Cas9 (Table 2.3).

Table 2.3. Comparing gRNA delivery methods. *Spy*Cas9- and *Fn*Cas12a-mediated DNA editing efficiency in IMX1905 transformed with different delivery methods for the gRNA-expression cassette. **A)** *in vitro* pre-assembled plasmids; **B)** *in vivo* assembly after co-transformation with gRNA expression cassette and marker backbone with homology flanks; **C)** co-transformation of gRNA expression cassette with circular empty plasmid (pGGKd018); **D)** co-transformation of gRNA expression cassette with split empty plasmid (pGGKd018). The gRNA cassettes specific for *Spy*Cas9 and *Fn*Cas12a editing are depicted at the left of the table (p, promoter; DR, direct repeat; t, terminator) and their respective length in bp is reported. These were compared for expression under the RNAPolIII-dependent *SNR52p* (pUDR585 for *Spy*Cas9, pUDR484 for *Fn*Cas12a), the T7RNAP-dependent 17bp-long S.T7p (pUDR579 for *Spy*Cas9, pUDR488 for *Fn*Cas12a) or the T7RNAP-dependent 27bp-long L.T7p (pUDR581 for *Spy*Cas9, pUDR492 for *Fn*Cas12a). Editing efficiency is expressed as percentage of red colonies (*ade2*$^-$). Data represent the average and standard deviation of biological triplicates.

				ade2$^-$ frequency (%)			
				A. ***in vitro*** **assembly**	**B.** ***in vivo*** **assembly**	**C.** **circular plasmid**	**D.** **split plasmid**
							
	prom	term	Size of gRNA cassette (bp)				
gRNA cassette for **Spy**Cas9 	*SNR52*	*SUP4*	401	100.0 ± 0.0	90.7 ± 1.7	11.4 ± 4.0	23.8 ± 6.6
	S.T7	T7	166	0.0 ± 0.0	0.6 ± 0.4	--	--
	L.T7	T7	176	0.2 ± 0.2	1.2 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
gRNA cassette for *Fn*Cas12a 	*SNR52*	*SUP4*	359	100.0 ± 0.0	87.1 ± 4.5	12.3 ± 3.8	30.7 ± 5.2
	S.T7	T7	124	4.6 ± 0.2	7.8 ± 3.8	--	--
	L.T7	T7	134	8.2 ± 3.2	20.1 ± 4.9	5.7 ± 1.9	6.7 ± 2.9

The most popular method for gRNA delivery is via *in vitro* assembly of the gRNA expression cassette on a plasmid, and transformation of this circular plasmid to yeast. As expected, expression of the gRNA cassette from *SNR52p* using this delivery method led to high efficiency in editing of the *ADE2* gene in all colonies tested with both *Spy*Cas9 and *Fn*Cas12a (Table 2.3A). Conversely, T7RNAP^{K276R}-based gRNA expression resulted in extremely low editing efficiency with *Fn*Cas12a (4.6% ± 0.2% for the short and 8.2% ± 3.2% for the long T7 promoter) and null or negligible editing with *Spy*Cas9 (Table 2.3A). Next to delivering a ready-made gRNA plasmid, two parts, one carrying the gRNA and the other the selection marker, were transformed into yeast. These two parts were flanked by 60 bp homologous sequences to enable *in vivo* circularization upon transformation. This delivery method enabled the transient availability of the gRNA cassette as linear DNA fragment. While reducing the editing efficiency for *pSNR52*-based gRNA expression by ca. 10%, this method slightly increased *ADE2* editing by T7RNAP^{K276R}-mediated expression of gRNAs for both *Spy*Cas9 and *Fn*Cas12a (Table 2.3B). Editing by *Spy*Cas9 remained extremely low (around 1%), while up to 20% of the colonies displayed the disruption of *ADE2* by *Fn*Cas12a (Table 2.3B). Next, to test whether the gRNA could be solely expressed from a linear DNA molecule, the gRNA cassette was delivered as double-stranded DNA fragment. A plasmid carrying a selectable marker was transformed into yeast in parallel for selection purposes. With this delivery method, editing efficiency with RNAPolIII-mediated gRNA expression was dramatically reduced to ca. 10% with both *Spy*Cas9 and *Fn*Cas12a (Table 2.3C). Editing efficiency for T7RNAP^{K276R}-based gRNA expression was also reduced, but still detectable at around 6% when using *Fn*Cas12a. It has been shown that the efficiency of *Spy*Cas9-mediated DNA editing can be increased by supplying a split plasmid during transformation, presumably by offering a selective advantage to cells that are proficient in homology-directed repair (Horwitz *et al.*, 2015). Accordingly, a twofold increase in *ADE2* editing efficiency was measured with both *Spy*Cas9 and *Fn*Cas12a when the gRNAs were transcribed by RNAPolIII (Table 2.3CD). However, using a circular or a split plasmid did not affect DNA editing for T7RNAP^{K276R}-expressed gRNAs (Table 2.3CD). The split marker approach combined with linear DNA delivery of the gRNA described in Table 2.3D was nonetheless kept for all the following experiments.

Altogether, these data demonstrate that gRNAs can be expressed from circular and linear DNA using the T7RNA^{K276R} polymerase in *S. cerevisiae*. Additionally, *Fn*Cas12a leads to higher DNA editing efficiency than *Spy*Cas9 when guided by T7RNAP^{K276R}-expressed gRNAs. In all experiments, the long T7 promoter consistently led to two- to threefold higher editing efficiencies than the short T7 promoter (Table 2.3AB), as a possible consequence of the higher T7RNAP-promoter complex stability (Tang *et al.*, 2005). While delivery of gRNAs in the form of short linear DNA fragments enabled DNA editing, the observed editing efficiencies were low and required further optimization to turn the gEL DNA approach into an attractive and competitive DNA editing technique.

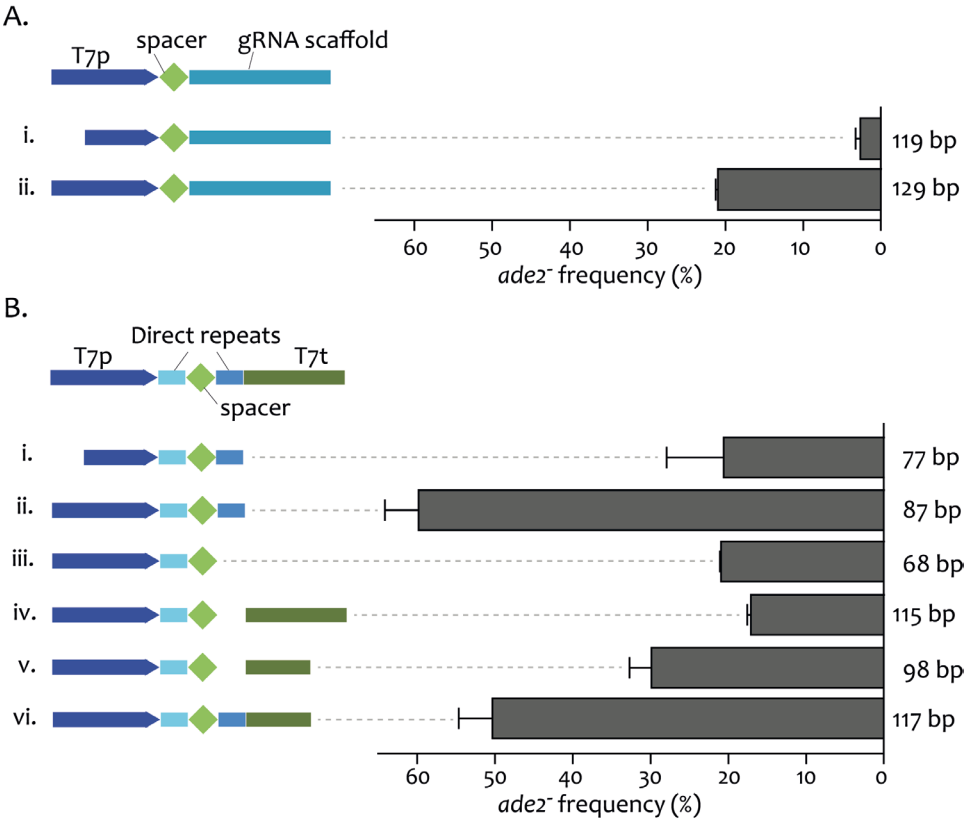


Figure 2.4. Optimization of *Spy*Cas9 and *Fn*Cas12a gDNA design. Editing efficiency of *ADE2* in strain IMX1905 transformed with gDNAs for cloning-free, T7RNAP-driven expression of gRNA. **A)** gDNA configurations for *Spy*Cas9-mediated genome editing and respective editing efficiencies. **B)** gDNA configurations for *Fn*Cas12a-mediated genome editing and their respective editing efficiencies. The size of each gDNA is specified on the right of the respective graph bar. Editing efficiency is expressed as percentage of red colonies (*ade2*$^-$) over the total number of colonies. Values represent the average and standard deviations of data obtained from three independent biological replicates.

Improving the efficiency of the T7RNAP^{K276R}–based gEL DNA technique by optimizing the gDNA design

Aiming for cloning-free genome editing, the gEL DNA technique relies on the simple utilization of customized double-stranded DNA oligos (referred to as gDNAs) for the *in vivo* T7RNAP^{K276R}-mediated expression of gRNAs. To reduce synthesis costs and increase compatibility with high-throughput strain construction, the size of the gDNA should be as small as possible and should not exceed 120 nt, the standard size limit of commercial, custom-made oligomers. In this respect, *Fn*Cas12a presents a clear advantage as its gRNAs consists of a smaller structural part (DR) than the one required for DNA targeting by *Spy*Cas9 (gRNA scaffold). Consequently, the small size of *Fn*Cas12a gRNAs gives more flexibility in gDNA design regarding length of T7

transcriptional elements and presence of terminal DR or T7 terminator. Conversely, a minimal gDNA configuration for *Spy*Cas9-mediated editing containing the S.T7p and the chimeric gRNA is 119-nt long (Figure 2.4), which does not leave room for additional, potentially useful parts such as a longer T7 promoter or a T7 terminator. As previously, a triplet of guanine was appended to the T7 promoter for all tested gDNA.

For *Spy*Cas9, two gDNA configurations were tested, one with the short and one with the long T7 promoter, followed by the *ADE2.y* spacer and the gRNA scaffold (Figure 2.4A). As compared to the design presented in Table 3, no T7 terminator was added at the end of the gDNA. While the pre-annealed S.T7p gDNA can be directly transformed into IMX1905, the longer L.T7p gDNA (129 bp) had to be obtained by a preliminary PCR reaction using two primers with overlapping homologies (see Materials and Methods section 2.2). Both gEL DNA configurations did enable *Spy*Cas9-mediated DNA editing, marginal for the S.T7p (2.6% editing efficiency, Figure 2.4A-i), but substantial for the L.T7p (21% editing efficiency, Figure 2.4A-ii).

Six different gDNA configurations for the *ADE2-3* target were tested for *Fn*Cas12a-mediated editing, differing in the size of T7 promoter and terminator as well as in the addition of a terminal DR and a T7 terminator (Figure 2.4B). After simple pre-annealing of two complementary oligos *in vitro*, each gDNA was directly transformed into strain IMX1905. These data revealed that the terminal DR is important for efficient editing of *ADE2* irrespective to the presence of the T7 terminator, and that the presence of a T7 terminator is not required (Figure 2.4B). These results also further confirmed that the long version of the T7 promoter markedly increased DNA editing efficiency (Figure 2.4B-i-ii). This design optimization enabled an increase of the DNA editing efficiency to 60%, relying on the very simple transformation of yeast with an 87 nt-long oligonucleotide. This simple and efficient design, represented in Figure 2.4B-ii, was implemented for the rest of the work with *Fn*Cas12a.

The editing efficiencies shown in Figure 2.4 were substantially higher for both *Spy*Cas9 and *Fn*Cas12a than those reported in Table 2.3D, in which a similar approach with linear gDNA delivery together with a split plasmid was also used. This increased efficiency most probably resulted from the higher amount of gDNA supplied to the transformation mix used for the experiments presented in Figure 2.4 (300 to 400-fold higher). The increased editing efficiency combined with the higher abundance of gDNA suggests that a greater supply of the gDNA to the cell might be a key element for genome editing using gEL DNA.

Improving the efficiency of the gEL DNA technique by optimizing sequence and expression levels of the T7RNAP

To further explore whether gDNA, and consequently gRNA availability might be limiting editing efficiency, gDNA transcription efficiency by the T7RNAP was explored.

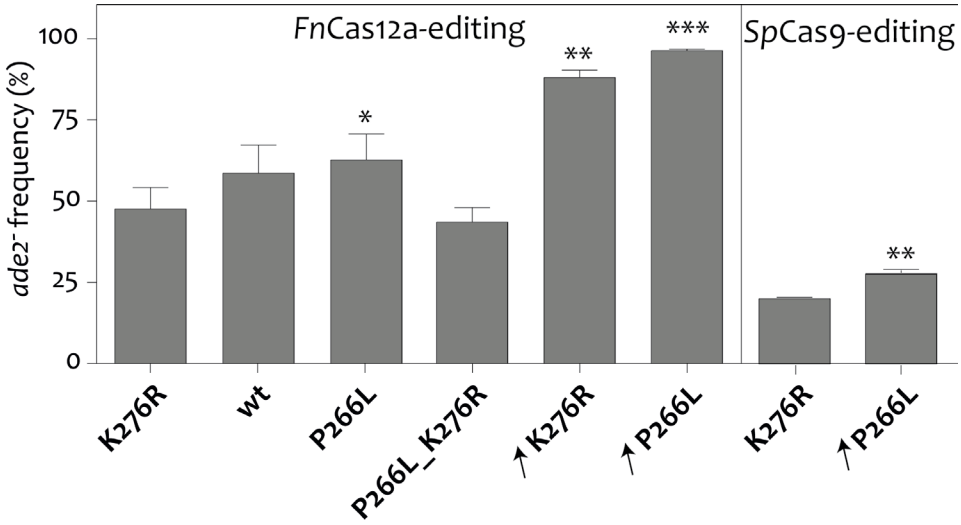


Figure 2.5. Figure 5 Comparison of *Spy*Cas9 and *Fn*Cas12a editing efficiency with T7RNAP variants. Efficiency of *ADE2* editing by *Fn*Cas12a- or *Spy*Cas9-mediated gEL DNA in T7RNAP mutant and/or overexpression strains: IMX1905 (K276R); IMX2031 (wild-type, wt); IMX2032 (P266L); IMX2030 (P266L and K276R); IME459 (K276R overexpression, ↗K276R); and IME475 (P266L overexpression, ↗P266L). For *Fn*Cas12a, transformed gDNA corresponds to annealed 15093 & 15094 oligos. For *Spy*Cas9, transformed gDNA was obtained through PCR using overlapping primers 16745 & 16746. Editing efficiency is expressed as percentage of red colonies (*ade2*$^-$). Values represent the average and standard deviations of data obtained from independent biological duplicates. *p < 0.05; **p < 0.025; ***p < 0.001. Student's t-test was calculated compared to respective control strain IMX1905 (K276R).

To this end, three additional T7RNAP variants were tested. All three variants were constructed from IMX1905, by inserting point mutations in the T7RNAP^{K276R} gene. In the first variant the K276R mutant was reverted into the wild-type T7RNAP (T7RNAP^{WT}, strain IMX2031). The second variant carried the P266L mutation, known to reduce abortive transcription *in vitro* (Guillerez *et al.*, 2005) (T7RNAP^{P266L}, strain IMX2032) and the third variant carried the two mutations (T7RNAP$^{P266L, R276K}$, strain IMX2030). When tested with *Fn*Cas12a, all four variants enabled DNA editing with a significantly higher efficiency for T7RNAP^{P266L} (Figure 2.5), while T7RNAP^{K276R} and T7RNAP showed the lowest DNA editing efficiency (Figure 2.5), suggesting that the K276R substitution is deleterious for T7RNAP transcription efficiency.

To further enhance gDNA transcription efficiency, the expression level of the T7RNAP was increased. The strains IME459 and IME475 were constructed by transformation with episomal plasmids harbouring the T7RNAP^{R276K} and the T7RNAP^{P266L} variants, respectively. While expression of T7RNAP from a single, integrated gene copy did not affect growth of *S. cerevisiae* (Figure 2.3), expression from episomal vectors significantly reduced the growth rate when compared to a control strain carrying an empty episomal vector (0.29 ± 0.00 h^{-1} for IME459, 0.26 ± 0.01 h^{-1} for IME475 and 0.31 ± 0.01 h^{-1} for the control strain IME460, Figure 2.3; Table 2.1). Overexpression of either

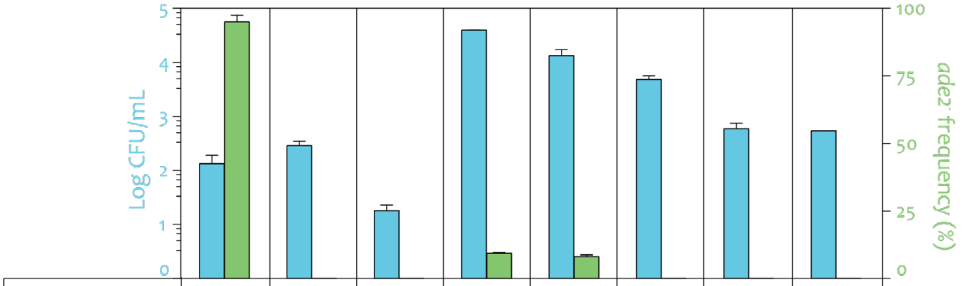
T7RNAP strongly increased the DNA editing efficiency by *Fn*Cas12a, approaching 100% when using T7RNAP^{P266L} (Figure 2.5). Overexpression of T7RNAP^{P266L} also increased DNA editing efficiency by *Spy*Cas9, as compared to a single copy of T7RNAP^{K276R}, but to a lesser extent (increase by 10%, Figure 2.5). The gEL DNA approach remained much more efficient with *Fn*Cas12a than with *Spy*Cas9 (maximum efficiencies of 96% and 29%, respectively, Figure 2.5). Finally, to test whether the efficiency of gEL DNA was sensitive to the location and sequence of the edited site, using these optimized conditions, three new sites were selected in non-coding regions and were tested for *Fn*Cas12a-mediated targeting with the T7RNAP^{P266L}-overexpression strain (IME475). The high efficiency of single gEL DNA editing was confirmed with efficiency between 90 and 100% for these three loci (XI-3 (Mikkelsen *et al.*, 2012), XVI-1 and YPRCτ3 loci, Figure S4). Altogether, these results revealed that the expression levels of the T7RNAP, and therefore most likely gRNA availability, play a key role for successful DNA editing by *Fn*Cas12a in the gEL DNA system.

Assessment on the components required for efficient DNA editing using gEL DNA

In the approach described above, gEL DNA requires five components: the T7RNAP and *Fn*Cas12a already present in the transformed strain, and the gDNA cassette, repair DNA and split plasmid carrying the selection marker, delivered as linear fragments during transformation. Control experiments in which these components where systematically omitted confirmed that all five components are required for efficient DNA editing using the gEL DNA method (Figure 2.6; Figure S5). Editing was completely abolished in the absence of targeting gDNA and of repair DNA, as well as if the targeting gDNA was replaced by a non-targeting variant, with or without repair DNA. The supply of the split plasmid is understandably not essential for DNA editing using the gEL DNA method, but is key for the selection of edited transformants, as its absence resulted in an abundance of colonies on the plates and very low editing efficiency (ca. 8%). It is however remarkable that, even in the absence of selection marker, correctly edited transformants could be found. It is interesting to note that DNA editing was observed in the absence of T7RNAP, albeit with extremely low efficiency (ca. 8%). This editing was however abolished when *Fn*Cas12a was also omitted. As the presence of targeting gDNA is necessary for DNA editing by *Fn*Cas12a (Figure 2.6, no editing in the absence of targeting gDNA), this T7RNAP-independent editing might be explained by low-level transcription of the supplied gDNA by one of the native yeast polymerases or by guiding of *Fn*Cas12a by single-stranded gDNA present as contamination of the double-stranded gDNA stock, although both hypotheses seem unlikely. Overall, *Fn*Cas12a, T7RNAP, gDNA, repair DNA and selection plasmid are essential for maximum DNA editing efficiency by gEL DNA.

gEL DNA enables *Fn*Cas12a-mediated multiplex genome editing in *S. cerevisiae*

To test for multiplex genome editing, four gRNAs targeting *CAN1*, *HIS4*, *PDR12* and *ADE2*,



ADE2 gDNA	+	-	+	+	+	+	-	-
Δ*ADE2* repair	+	+	-	+	+	+	+	-
Selection plasmid	+	+	+	-	+	+	+	+
nt gDNA	-	-	-	-	-	-	+	+
T7RNAP	+	+	+	+	-	-	+	+
Cas12a	+	+	+	+	+	-	+	+

Figure 2.6. CFU and *ADE2* editing efficiencies with different combinations of the components of the gEL DNA system. *S. cerevisiae* strains carrying *Fn*Cas12a and T7RNAP (IME475), *Fn*Cas12a alone (IMX1714), or control strain (CEN.PK-113.7D) were transformed with *ADE2* or non-targeting (nt) gDNAs, alternatively omitting repair DNA for *ADE2* deletion or split pGGKd018 plasmid for selection. Results of cell counts are presented as log CFU mL^{-1} culture. Editing efficiency is expressed as percentage of red colonies (*ade2*$^-$), which were further verified by PCR (Figure S5).

previously shown to lead to 100% DNA editing efficiency by *Fn*Cas12a when expressed from a RNAPolIII promoter, were selected ((Swiat *et al.*, 2017), Figure 2.7A). As done for the *ADE2-3* target used for singleplex gEL DNA, these additional gRNAs were shortened to a 19 bp-long spacer as compared to the previously described plasmid-based constructs (Swiat *et al.*, 2017). Oligos carrying the gDNA design shown in Figure 4B-ii were ordered for each gRNA (Table S1) and transformed in duplex or quadruplex to IME475 overexpressing the T7RNAP^{P266L}. Duplex targeting of *ADE2* and *HIS4* revealed that a vast majority of tested clones were edited (14 out of 16) and that 63% of the clones carried a double deletion (Figure 2.7B). Out of the clones with a single edit, none carried a single *HIS4* deletion, while duplex editing with *ADE2* was clearly a frequent event (Figure S6). Quadruplex targeting resulted in a substantial fraction of clones without any editing (34%, Figure 2.7C). The fraction of clones with a single editing event was very similar for duplex and quadruplex editing (25 and 30%, respectively). 23% and 13% of the clones carried double and triple edits, respectively, and quadruplex editing was not observed (Figure 2.7C). Remarkably, none of the tested clones displayed editing in *CAN1* (Figure S7), suggesting that the *CAN1-4* gRNA failed to guide *Fn*Cas12a to the targeted site. This lack of targeting might be explained by the fact that the *CAN1-4* gRNA contained an additional guanine triplet and was six nucleotides shorter than the *CAN1-4* gRNA originally tested by Swiat and co-workers. To test this hypothesis, the *CAN1-4* gDNA (GGG at 5' and a 19 bp-long spacer) was expressed from a plasmid with the *SNR52* promoter and tested for editing efficiency. Out of eight selected colonies,

none resulted in a *CAN1* deletion (Figure S8), a complete loss in editing efficiency that is likely due to the disruption of the gRNA stem-loop structure (Table S3). A new *CAN1* spacer with a predicted secondary structure displaying the gRNA stem-loop was therefore selected for *CAN1* targeting (*CAN1-3* (Swiat *et al.*, 2017), Table S3). Expressed from a plasmid with the *SNR52* promoter, *CAN1-3* led to 100% *CAN1* editing with *Fn*Cas12a (Figure S8). However, when tested for multiplexing using the gEL DNA, *CAN1-3* rarely led to editing of *CAN1* by *Fn*Cas12a (Figure 2.7D). A single *CAN1* editing event was observed out of 30 clones tested (Figure S9) and, remarkably, this event was concomitant with the editing of the three other targets, leading to a single clone with quadruple DNA editing (Figure 2.7D). In the quadruplex editing experiments with *CAN1-4* and *CAN1-3*, the fraction of clones with single, double and triple DNA editing was comparable (roughly 30, 25 and 10% respectively, Figure 2.7CD).

Following the approach described by Swiat and co-workers, two cRNA arrays were tested for quadruplex genome editing. Both plasmids carried the *HIS4-4*, *ADE2-3* and *PDR12-3* gRNAs, but pUDE735 expressed *CAN1-4* (Figure S7) while pUDR692 expressed *CAN1-3* (Figure S9). As previously observed, the number of colonies obtained after transformation was extremely low (below ten colonies), as compared to the number of colonies obtained for quadruplex editing with the gEL DNA approach (over 150 colonies).

Construction and validation of a portable gEL DNA toolkit

The orthogonality of the T7RNAP-based gEL DNA system has great potential for other organisms. To demonstrate transportability, all-in-one multicopy plasmids carrying both T7RNAP and *Fncas12a* were constructed (pUDE1083, pUDE1084 and pUDE1087, Table 2.2). Both proteins have been shown to reduce growth rate when expressed individually at high level from multicopy plasmids (this work for T7RNAP and (Swiat *et al.*, 2017) for *Fncas12a*), simultaneous high-level expression of these two proteins might therefore be detrimental for the yeast strains. The results above show that the efficiency of DNA editing by gEL is sensitive to T7RNAP abundance, it was therefore decided to keep the same promoter for T7RNAP expression (*pTDH3*) but to tune the expression of *Fncas12a* by using three constitutive promoters spanning a broad range of strengths: *pREV1* with low expression (resulting in strain IME646), *pPFK1* with intermediate expression (strain IME640) and the strong *pTEF1* (strain IME641). As expected, co-expression of T7RNAP and *Fncas12a* decreased the specific growth rate as compared to strains expressing T7RNAP or *Fncas12a* alone and to the control strain (ca. 20% decrease, Figure S10), however strains with different promoter strengths for *Fncas12a* expression displayed similar growth rates (specific growth rate of 0.26 ± 0.01 h^{-1} for IME640, 0.25 ± 0.01 h^{-1} for IME641 and 0.25 ± 0.01 h^{-1} for IME646; Figure S10). In the absence of promoter-dependent phenotypic effect, the strain expressing *Fncas12a* under the control of the strongest, *pTEF1* promoter (IME641) was selected to test the

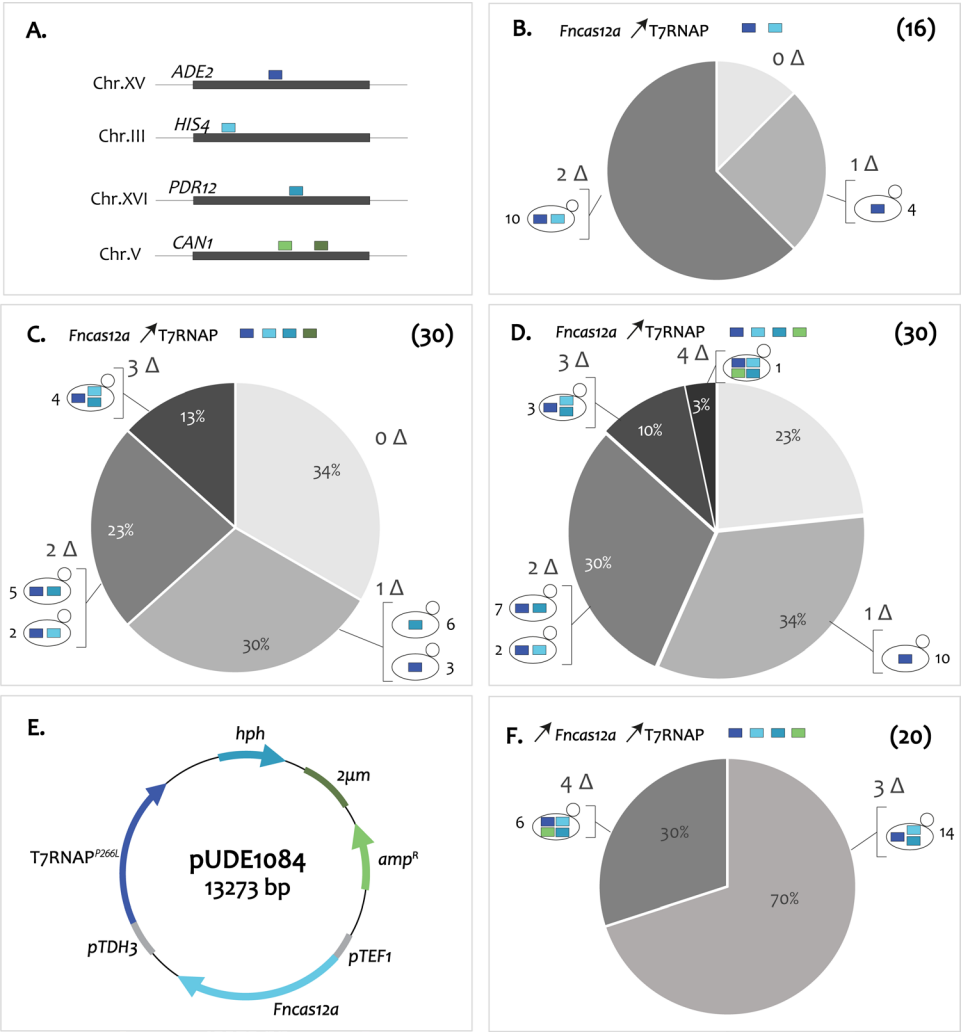


Figure 2.7. Multiplex genome editing by *Fn*Cas12a using the gEL DNA approach. **A)** Targeted sites for deletion of *ADE2* (*ADE2-3*, dark blue), *HIS4* (*HIS4-4*, light blue), *PDR12* (*PDR12-3*, turquoise), and *CAN1* (*CAN1-4*, light green; *CAN1-3*, dark green). **B)** IME475 (T7RNAP overexpression, ↗T7RNAP) transformants obtained from double gDNA delivery: *ADE2-3* and *HIS4-4*. **C)** Transformants of IME475 using four gDNAs: *ADE2-3*, *HIS4-4*, *PDR12-3*, and *CAN1-4*. **D)** Transformants of IME475 using four gDNAs: *ADE2-3*, *HIS4-4*, *PDR12-3*, and *CAN1-3*. **E)** Plasmid map of the exportable gEL DNA plasmid pUDE1084. **F)** Fraction of selected colonies upon transformation of IME641 (*Fncas12a* and T7RNAP overexpression, ↗*Fncas12a* ↗T7RNAP) with four gDNAs: *ADE2-3*, *HIS4-4*, *PDR12-3*, and *CAN1-3*. Number of verified clones is indicated in parentheses, and diagnostic PCRs are reported in Figures S6, S7, S9, S12, and S13. Zero (0Δ), single (1Δ), double (2Δ), triple (3Δ), or quadruple (4Δ) deletions are indicated at the outside ends for each fraction. Types of obtained deletions are specified by the respective colour of the target. The total number of tested colonies are also stated next to each depiction in parentheses.

DNA editing efficiency of the portable gEL DNA system (plasmid map in Figure 2.7E). Singleplex editing of *ADE2* revealed similar efficiencies between the integrated and portable systems (above 95 % ± 1% of edited colonies, Figure S11). However, the portable system proved to be substantially superior for duplex and quadruplex editing (Figure 2.7F, Figure S12, Figure S13) with 100% and 30% efficiency for duplex and quadruplex editing, respectively. Remarkably, the remaining 70% of the colonies transformed for quadruplex editing displayed triplex editing, with *CAN1* systematically unedited (Figure 2.7F). The plasmid-based gEL DNA approach is therefore highly efficient for singleplex and multiplex editing. It does not require *a priori* modification of target strain and is therefore a promising tool to be used in other yeasts, or even other organisms upon construction of compatible T7RNAP and *Fn*Cas12a-expressing plasmids.

2.4. Discussion

The future of CRISPR-Cas-based genome editing heads towards the development of fast and low-cost methodologies for strain construction. The gEL DNA approach presented in this study expands the CRISPR-Cas genome editing toolbox of *S. cerevisiae* with an entirely cloning-free and very efficient strategy for single or double genetic modification in *S. cerevisiae*. By simply transforming pre-annealed 87-long, complementary DNA oligonucleotides into competent yeast cells, cost and time of strain construction can be reduced to the bare minimum. Any chosen gRNA cassette can be delivered independently or in combination with other gRNA cassettes, making this technique very versatile and highly suitable for high-throughput, combinatorial strain construction. Akin to other CRISPR-based techniques for genome engineering, increasing the number of simultaneously targeted sites strongly affects the efficiency of multiplexed gEL DNA or the viability of cells in terms of CFU on transformation plate (Table S2, Figure 2.7) (Swiat *et al.*, 2017, Adiego-Pérez *et al.*, 2019). The results obtained in this study suggest that the editing efficiency can be further enhanced. For instance, increasing T7RNAP and gDNA abundance substantially increased singleplex gene editing (Figure 2.4, Figure 2.5), suggesting that gRNA abundance might be a key factor for efficient DNA editing. Measurement of gRNA abundance should be performed to confirm this hypothesis. However, measurement of these extremely short, transiently expressed RNAs during transformation is technically extremely challenging. While the toxicity of plasmid-borne T7RNAP expression showed that its abundance cannot be further increased in *S. cerevisiae*, the efficiency of the gEL DNA could be further enhanced by T7RNAP protein engineering or by expression of DNA-dependent RNAP variants from other bacteriophages (i.e. T3, SP6 or K11) that are able to transcribe from short promoters and from linear DNA templates (Jorgensen *et al.*, 1991, Rong *et al.*, 1999, Yoo & Kang, 2000). Another aspect to consider is the stability of the gDNA. While other methods deliver gRNA in the form of plasmids that are very stable *in vivo*, the linear nature of the gDNA make it prone to degradation by native exonucleases. Further

studies should explore the stability of gDNA and gRNA during transformation and test whether chemical stabilization of the linear gDNA (by phosphorothioate derivatives or 2'-ribose modification for instance (Leonetti *et al.*, 1991, Fisher *et al.*, 1993)) enhances gRNA availability and thereby DNA editing. These are therefore several promising avenues to further improve multiplex DNA editing with the gEL DNA approach.

Out of the eight gRNAs tested in this study, one failed to guide *Fn*Cas12a for gene editing. Remarkably, for this guide (*CAN1-4*), the folding prediction suggested the complete disruption of the direct repeat as a consequence of the 5'-addition of the guanine triplet, while the other seven guides displayed typical gRNA secondary structures with the required stem-loop structure (Figure 2.7, Table S3) (Swarts *et al.*, 2017). In agreement with these observations, a recent study about the *Fn*Cas12a-gRNA functionality suggests that the disruption of the direct repeat pseudoknot structure by pairing to the spacer sequence might lead to loss of gRNA targeting ability (Creutzburg *et al.*, 2020). Additionally, inhibition of the gRNA processing and consequently of *Fn*Cas12a activity seems to be due to the positional effect of a stable secondary structure flanking the direct repeat (Liao *et al.*, 2019). It has been recently advised that the terminator should be spaced-out by a 24 nt-long spacer to avoid steric effects with the pseudoknot formation and thereby allow correct gRNA folding (Creutzburg *et al.*, 2020). Our findings support these theories, since a gRNA flanked by the short, 30-nt T7 terminator sequence that lacks the stem-loop structure has a 1.8-fold higher *ADE2* editing efficiency than a gRNA with the longer, 47-nt T7 terminator (Figure 2.4B-iv-v). Prediction of the gRNA structure is therefore essential to optimize *Fn*Cas12a-based DNA editing with the gEL approach.

Despite efforts to improve editing with both *Spy*Cas9 and *Fn*Cas12a, the latter proved to be more efficient for DNA editing with the gEL DNA method. The causes for *Spy*Cas9's lower efficiency remain to be elucidated, but the observation that increasing T7RNAP abundance hardly affects DNA editing by *Spy*Cas9 (increased by 1.4%; Figure 2.5) suggests that gRNA abundance is not the factor impairing *Spy*Cas9 activity. While the length of the gDNA might be another obstacle for *Spy*Cas9 implementation with the gEL DNA approach, it could be overcome by expressing the tracrRNA separately from the gRNA (Bao *et al.*, 2015).

In conclusion, the gEL DNA methodology is not only an extremely valuable tool for genome editing in *S. cerevisiae*, but has a yet greater potential thanks to its portability to other organisms. Expression of gRNAs using the host machinery or *in vivo* burden of gRNA-expression plasmids can present serious obstacles for CRISPR-Cas9-based editing (Gao & Zhao, 2014, Wagner *et al.*, 2014, Gorter de Vries *et al.*, 2017, Lee *et al.*, 2017, Juergens *et al.*, 2018). By introducing a T7RNAP and gDNA oligos, the gEL DNA approach dissociates gRNA production from the host polymerase and from plasmid templates, thereby entirely removing these obstacles.

Data availability

Supplementary information can be accessed via the online publication.

Acknowledgment

We thank Melanie Wijsman and Ewout Knibbe for constructing strains IMX1714 and IMX1752, respectively, Sofia Dashko for cloning plasmid pGGKd034, and Marcel van den Broek for the sequence alignment of the T7 promoter to the whole *S. cerevisiae* genome.

2.5. References

Adiego-Pérez B, Randazzo P, Daran JM, Verwaal R, Roubos JA, Daran-Lapujade P & van der Oost J (2019) Multiplex genome editing of microorganisms using CRISPR-Cas. *FEMS Microbiology Letters* **366**.

Baldi N, Dykstra JC, Luttik MAH, Pabst M, Wu L, Benjamin KR, Vente A, Pronk JT & Mans R (2019) Functional expression of a bacterial α-ketoglutarate dehydrogenase in the cytosol of *Saccharomyces cerevisiae*. *Metabolic Engineering* **56**: 190-197.

Bao Z, Xiao H, Liang J, Zhang L, Xiong X, Sun N, Si T & Zhao H (2015) Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae*. *Acs Synthetic Biology* **4**: 585-594.

Bellaousov S, Reuter JS, Seetin MG & Mathews DH (2013) RNAstructure: Web servers for RNA secondary structure prediction and analysis. *Nucleic Acids Research* **41**: 471-474.

Boonekamp FJ, Dashko S, van den Broek M, Gehrmann T, Daran J-M & Daran-Lapujade P (2018) The genetic makeup and expression of the glycolytic and fermentative pathways are highly conserved within the *Saccharomyces* genus. *Frontiers in genetics* **9**: 504-504.

Creutzburg SCA, Wu WY, Mohanraju P, Swartjes T, Alkan F, Gorodkin J, Staals RHJ & der Oost JV (2020) Good guide, bad guide: spacer sequence-dependent cleavage efficiency of Cas12a. *Nucleic Acids Research* **6**:3228-3243.

de Kok S, Nijkamp JF, Oud B, Roque FC, de Ridder D, Daran JM, Pronk JT & van Maris AJ (2012) Laboratory evolution of new lactate transporter genes in a *jen1Δ* mutant of *Saccharomyces cerevisiae* and their identification as *ADY2* alleles by whole-genome resequencing and transcriptome analysis. *FEMS Yeast Research* **12**: 359-374.

DiCarlo JE, Norville JE, Mali P, Rios X, Aach J & Church GM (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research* **41**: 4336-4343.

Dorfman BZ (1969) The isolation of adenylosuccinate synthetase mutants in yeast by selection for constitutive behavior in pigmented strains. *Genetics* **61**: 377-389.

Dower K & Rosbash M (2002) T7 RNA polymerase-directed transcripts are processed in yeast and link 39 end formation to mRNA nuclear export. *RNA* **8**: 686-697.

Easmin F, Hassan N, Sasano Y, Ekino K, Taguchi H & Harashima S (2019) gRNA-transient expression system for simplified gRNA delivery in CRISPR/Cas9 genome editing. *Journal of Bioscience and Bioengineering* **128**: 373-378.

Entian KD & Kotter P (2007) Yeast genetic strain and plasmid collections. *Method Microbiology* **36**: 629-666.

Ferreira R, Skrekas C, Nielsen J & David F (2018) Multiplexed CRISPR/Cas9 genome editing and gene regulation using Csy4 in *Saccharomyces cerevisiae*. *ACS Synthetic Biology* **7**: 10-15.

Fisher TL, Terhorst T, Cao X & Wagner RW (1993) Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells. *Nucleic Acids Research* **21**: 3857-3865.

Flagfeldt D, Siewers V, Huang L & J N (2009) Characterization of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*. *Yeast* **26**: 545–551.

Gao Y & Zhao Y (2014) Self-processing of ribozyme-flanked RNAs into guide RNAs *in vitro* and *in vivo* for CRISPR-mediated genome editing. *Journal Integrative Plant Biology* **56**: 343-349.

Generoso WC, Gottardi M, Oreb M & Boles E (2016) Simplified CRISPR-Cas genome editing for *Saccharomyces cerevisiae*. *Journal of Microbiol Methods* **127**: 203-205.

Gietz RD & Schiestl RH (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols* **2**: 31-34.

Gorter de Vries AR, de Groot PA, van den Broek M & Daran J-MG (2017) CRISPR-Cas9 mediated gene deletions in lager yeast *Saccharomyces pastorianus*. *Microbial Cell Factories* **16**: 222.

Guillerez J, Lopez P, Proux F, Launay H & Dreyfus M (2005) A mutation in T7 RNA polymerase that facilitates promoter clearance. *PNAS* **102**: 5958–5963.

Hassing E-J, de Groot PA, Marquenie VR, Pronk JT & Daran J-MG (2019) Connecting central carbon and aromatic amino acid metabolisms to improve de novo 2-phenylethanol production in *Saccharomyces cerevisiae*. *Metabolic Engineering* **56**: 165-180.

Horwitz AA, Walter JM, Schubert MG, Kung SH, Hawkins K, Platt DM, Hernday AD, Mahatdejkul-Meadows T, Szeto W, Chandran SS & Newman JD (2015) Efficient multiplexed integration of synergistic elleles and metabolic pathways in yeasts via CRISPR-Cas. *Cell Systems* **1**: 88-96.

Imburgio D, Rong M, Ma K & McAllister WT (2000) Studies of promoter recognition and start site selection by T7 RNA polymerase using a comprehensive collection of promoter variants. *Biochemistry* **39**: 10419-10430.

Jakočiūnas T, Bonde I, Herrgård M, Harrison SJ, Kristensen M, Pedersen LE, Jensen MK & Keasling JD (2015) Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae*. *Metabolic Engineering* **28**: 213-222.

Jorgensen ED, Durbin RK, Risman SS & McAllister WT (1991) Specific contacts between the bacteriophage T3, T7, and SP6 RNA polymerases and their promoters. *Journal of Biological Chemistry* **266**: 645-651.

Juergens H, Varela JA, Gorter de Vries AR, Perli T, Gast VJM, Gyurchev NY, Rajkumar AS, Mans R, Pronk JT, Morrissey JP & Daran J-MG (2018) Genome editing in *Kluyveromyces* and *Ogataea* yeasts using a broad-host-range Cas9/gRNA co-expression plasmid. *FEMS Yeast Research* **18**.

Langmead B, Trapnell C, Pop M & Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10**: R25.

Lee K, Conboy M, Park HM, et al. (2017) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA *in vivo* induces homology-directed DNA repair. *Nature Biomedical Engineering* **1**: 889-901.

Lee ME, DeLoache WC, Cervantes B & Dueber JE (2015) A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synthetic Biology* **4**: 975-986.

Leonetti JP, Mechti N, Degols G, Gagnor C & Lebleu B (1991) Intracellular distribution of microinjected antisense oligonucleotides. *Proceedings of the National Academy of Sciences* **88**: 2702-2706.

Li Z-H, Wang F-Q & Wei D-Z (2018) Self-cloning CRISPR/Cpf1 facilitated genome editing in *Saccharomyces cerevisiae*. *Bioresources and Bioprocessing* **5**: 36.

Li ZH, Liu M, Lyu XM, Wang FQ & Wei DZ (2018) CRISPR/Cpf1 facilitated large fragment deletion in *Saccharomyces cerevisiae*. *Journal of Basic Microbiology* **58**: 1100-1104.

Liao C, Slotkowski RA, Achmedov T & Beisel CL (2019) The *Francisella novicida* Cas12a is sensitive to the structure downstream of the terminal repeat in CRISPR arrays. *RNA Biology* **16**: 404-412.

Mans R, van Rossum HM, Wijsman M, Backx A, Kuijpers NG, van den Broek M, Daran-Lapujade P, Pronk JT, van Maris AJ & Daran JM (2015) CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Research* **15**.

Meijnen JP, Randazzo P, Foulquie-Moreno MR, et al. (2016) Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. *Biotechnol Biofuels* **9**: 5.

Mikkelsen MD, Buron LD, Salomonsen B, Olsen CE, Hansen BG, Mortensen UH & Halkier BA (2012) Microbial production of indolylglucosinolate through engineering of a multi-gene pathway in a versatile yeast expression platform. *Metabolic Engineering* **14**: 104-111.

Milne N, Luttik MAH, Cueto Rojas HF, Wahl A, van Maris AJA, Pronk JT & Daran JM (2015) Functional expression of a heterologous nickel-dependent, ATP-independent urease in *Saccharomyces cerevisiae*. *Metabolic Engineering* **30**: 130-140.

Morse NJ, Wagner JM, Reed KB, Gopal MR, Lauffer LH & Alper HS (2018) T7 Polymerase expression of guide RNAs *in vivo* allows exportable CRISPR-Cas9 editing in multiple yeast hosts. *Acs Synthetic Biology* **7**: 1075-1084.

Rong M, Castagna R & McAllister WT (1999) Cloning and purification of bacteriophage K11 RNA polymerase. *Biotechniques* **27**: 690, 692, 694.

Ryan OW & Cate JH (2014) Multiplex engineering of industrial yeast genomes using CRISPRm. *Methods Enzymology* **546**: 473-489.

Ryan OW, Skerker JM, Maurer MJ, et al. (2014) Selection of chromosomal DNA libraries using a multiplex CRISPR system. *eLife* **3**.

Salazar AN, Gorter de Vries AR, van den Broek M, Wijsman M, de la Torre Cortés P, Brickwedde A, Brouwers N, Daran JG & Abeel T (2017) Nanopore sequencing enables near-complete *de novo* assembly of *Saccharomyces cerevisiae* reference strain CEN.PK113-7D. *FEMS Yeast Research* **17**.

Solis-Escalante D, Kuijpers NG, Bongaerts N, Bolat I, Bosman L, Pronk JT, Daran JM & Daran-Lapujade P (2013) amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. *FEMS Yeast Research* **13**: 126-139.

Swarts DC, van der Oost J & Jinek M (2017) Structural basis for guide RNA processing and seed-dependent DNA targeting by CRISPR-Cas12a. *Molecular Cell* **66**: 221-233 e224.

Swiat MA, Dashko S, den Ridder M, Wijsman M, van der Oost J, Daran JM & Daran-Lapujade P (2017) FnCpf1: a novel and efficient genome editing tool for *Saccharomyces cerevisiae*. *Nucleic Acids Research* **45**: 12585-12598.

Tang GQ, Bandwar RP & Patel SS (2005) Extended upstream A-T sequence increases T7 promoter strength. *Journal of Biological Chemistry* **280**: 40707-40713.

Verduyn C, Postma E, Scheffers WA & Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**: 501-517.

Verwaal R, Buiting-Wiessenhaan N, Dalhuijsen S & Roubos JA (2018) CRISPR/Cpf1 enables fast and simple genome editing of *Saccharomyces cerevisiae*. *Yeast* **35**: 201-211.

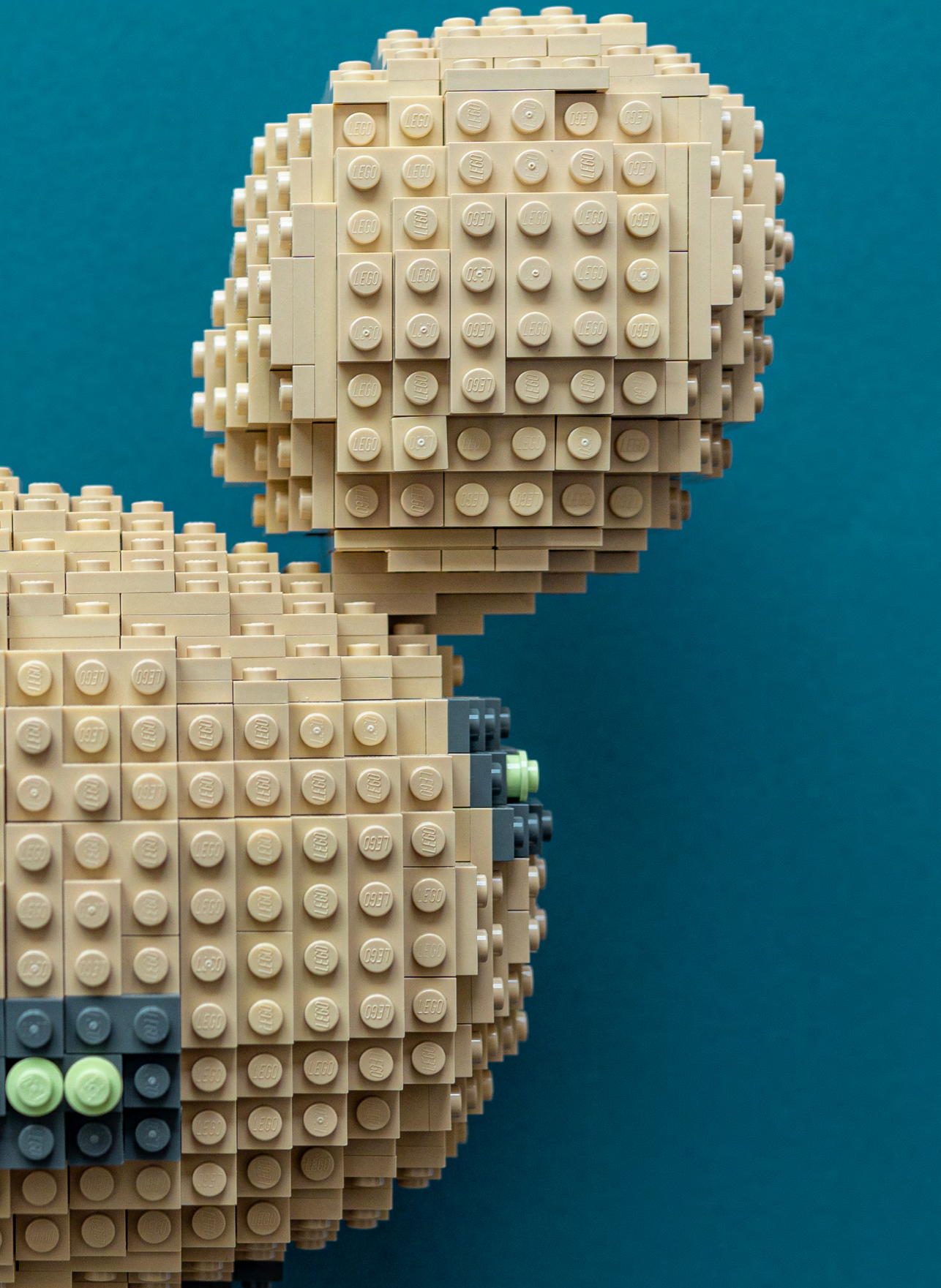
Wagner JC, Platt RJ, Goldfless SJ, Zhang F & Niles JC (2014) Efficient CRISPR-Cas9-mediated genome editing in Plasmodium falciparum. *Nature Methods* **11**: 915-918.

Walter JM, Chandran SS & Horwitz AA (2016) CRISPR-Cas-Assisted Multiplexing (CAM): simple same-day multi-locus engineering in yeast. *Journal of Cellular Physiology* **231**: 2563-2569.

Yoo J & Kang C (2000) Bacteriophage SP6 RNA polymerase mutants with altered termination efficiency and elongation processivity. *Biomolecular Engineering* **16**: 191-197.

Zhang Y, Wang J, Wang Z, Zhang Y, Shi S, Nielsen J & Liu Z (2019) A gRNA-tRNA array for CRISPR-

Cas9 based rapid multiplexed genome editing in *Saccharomyces cerevisiae*. *Nature Communications* **10**: 1053.



Chapter 3

Expanding the genome editing toolbox of *Saccharomyces cerevisiae* with the CRISPR-endonuclease *Er*Cas12a

Nicole X. Bennis, Jonah P. Anderson, Siebe M.C. Kok & Jean-Marc G. Daran

This chapter has been published in FEMS Yeast Research (2023)

Abstract

*Er*Cas12a is a class 2 type V CRISPR-Cas nuclease isolated from *Eubacterium rectale* with attractive fundamental characteristics, such as RNA self-processing capability, and lacks reach-through royalties typical for Cas nucleases. This study aims to develop a *Er*Cas12a-mediated genome editing tool applicable in the model yeast *Saccharomyces cerevisiae*. The optimal design parameters for *Er*Cas12a editing in *S. cerevisiae* were defined as a 21 nt spacer flanked by 19 nt direct repeats expressed from either RNApolII or III promoters, achieving near 100% editing efficiencies in commonly targeted genomic locations. To be able to transfer the *Er*Cas12a genome editing tool to different strain lineages, a transportable platform plasmid was constructed and evaluated for its genome editing efficiency. Using an identical crRNA expression design, the transportable *Er*Cas12a genome editing tool showed lower efficiency when targeting the *ADE2* gene. In contrast to genomic *Ercas12a* expression, episomal expression of *Ercas12a* decreases maximum specific growth rate on glucose, indicating *Er*Cas12a toxicity at high expression levels. Moreover, *Er*Cas12a processed a multi-spacer crRNA array using the RNA self-processing capability, which allowed for simultaneous editing of multiple chromosomal locations. *Er*Cas12a is established as a valuable addition to the genetic toolbox for *S. cerevisiae*.

3.1. Introduction

Originally studied for their role in prokaryotic adaptive immunity (Ishino *et al.*, 1987, Mojica *et al.*, 1993, Mojica *et al.*, 2005, Barrangou *et al.*, 2007, Brouns *et al.*, 2008, Marrafini & Sontheimer, 2009, Garneau *et al.*, 2010), clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems have since been studied for their use in genome editing (Sapranauskas *et al.*, 2011, Jinek *et al.*, 2012, Cong *et al.*, 2013, Mali *et al.*, 2013). In this application, precisely and efficiently introducing double stranded breaks (DSBs) is critical to activate and recruit the DNA repair machinery to the targeted location for precise genetic modifications. The freedom to design spacers at will makes the sequence-guided endonuclease function of CRISPR-Cas systems attractive for this purpose.

Genome editing strategies rely on a single effector Cas protein, consisting of a single crRNA-programmable multidomain, belonging to one of the class 2 CRISPR-systems. In contrast, class 1 CRISPR-systems rely on hetero multimer complexes, making them less attractive as genome editing tool (Makarova *et al.*, 2020). Applying CRISPR-Cas systems for gene editing is therefore based on the introduction of a class 2 Cas protein loaded with a CRISPR-RNA (crRNA). In native Archaeal and Prokaryotic hosts, the endonuclease-programming RNA can be composed of an RNA duplex resulting from the combination of a crRNA and a tracrRNA (Jinek *et al.*, 2012) or composed of a single RNA molecule (Zetsche *et al.*, 2015), like for Cas9 (class 2 type II) and Cas12a (class 2 type V-A), respectively. Upon complementarity of the spacer and protospacer and presence of the essential protospacer adjacent motif (PAM) (Garneau *et al.*, 2010), this ribonucleoprotein complex cleaves the DNA. For efficient application in heterologous hosts, the CRISPR class 2 type II system (Cas9) has been simplified by connecting the crRNA to the tracrRNA in a chimeric single guide RNA (sgRNA) (Jinek *et al.*, 2012). Targeted DSBs can be exploited to direct DNA repair mechanisms to the target locus. In cells with a functional homologous recombination (HR) machinery, adding a DNA repair fragment containing flanking homology sequences to the target site resolves the otherwise lethal dsDNA break, thereby incorporating the desired genetic modification (Capecchi, 1989).

Within the class 2 Cas proteins, the type II, which includes the Cas9 signature protein, was the first to be harnessed for gene editing applications (Jinek *et al.*, 2012) and has remained the dominant endonuclease for this purpose. While first applications were demonstrated in human cell lines, the Cas9 system has shown efficacy in a wide range of hosts (DiCarlo *et al.*, 2013, Feng *et al.*, 2013, Jiang *et al.*, 2013, Mizuno *et al.*, 2014, Juergens *et al.*, 2018). For effective endonuclease activity, the spacer sequence must be adjacent to a CRISPR-system-dependent PAM. The popular *Streptococcus pyogenes* Cas9 (*Spy*Cas9) recognizes the 5'-NGG-3' PAM, a sequence that occurs frequently enough to target editing events in most genes within a genome. However, the occurrence and location of PAM sequences may limit *in vivo* site-directed mutagenesis approaches

Table 3.1. Overview of reported *Er*Cas12a genome editing.

Organism	*Ercas12a* expression	crRNA	PAM (5' → 3')	DR length (bp)	Spacer length (bp)	Editing efficiency (%)	Reference
E. coli	unreported	unreported	YTTN	21	21	100%	Inscripta Inc.
S. cerevisiae	unreported	unreported	YTTN	36	21	66%	Inscripta Inc.
S. cerevisiae	Genomic expression, *pScPGK1*[a]	pUDP240 plasmid, RNApolII (*pScTDH3*[b]), HH&HDV, panARS origin of replication	TTTV	19	21	100%	This study
S. cerevisiae	pUDP293 plasmid (panARS origin of replication), *pScPGK1*[a]	pUDP293 plasmid, RNApolII (*pScTDH3*[b]), HH&HDV, panARS origin of replication	TTTV	19	21	31.5%	This study
Aspergillus nidulans, Aspergillus niger, Aspergillus aculeatus, Aspergillus oryzae	AMA1 plasmid *pAnTEF1*[c]	AMA1 plasmid RNApolIII (*pAoU6*[d])	YTTN	19	21	50%	(Jarczynska *et al.*, 2021)
Aspergillus species: Aspergillus nidulans, A. niger, A. oryzae and A. campestris	AMA1 plasmid *pAnTEF1*[c]	AMA1 plasmid RNApolIII (*U3*), glycine tRNA-based splicing	TTTN	35	21	unreported	(Vanegas *et al.*, 2022)
Rice and wheat protoplasts	Agrobacterium transferred T-DNA *pZmUbi-1*[e]	Episomal expression, RNApolII *pUbi-1*, HH&HDV	TTTN	35 or 21	20-24	66%	(Lin *et al.*, 2021)
Rice	Episomal expression, *pUbi-1*[e]	Episomal expression, RNApolII *pUbi-1*, HH&HDV	TTTV	unreported	19-23	89%	(Zhang *et al.*, 2021)

Table 3.1. (continued)

Organism	*Ercas12a* expression	crRNA	PAM (5' → 3')	DR length (bp)	Spacer length (bp)	Editing efficiency (%)	Reference
Mouse	Nucleofection (protein/RNA)	Nucleofection (RNA)	YTTN	35	21	81%	(Liu *et al.*, 2020)
Rat	Nucleofection (protein/RNA)	Nucleofection (RNA)	YTTN	35	21	25%	(Liu *et al.*, 2020)
Human cells	Nucleofection (protein/RNA)	Nucleofection (RNA)	YTTN	35	21	23%	(Liu et al., 2020)
Bacillus subtilis	Episomal expression, pGRAC	Episomal expression, *pVEG*	YTTN	35	21	100%	(Price *et al.*, 2020)
CHO cells	Transfection (protein, plasmid)	Transfection (RNA; plasmid, RNApolIII (pU6))	unreported	unreported	unreported	32%	(Rojek *et al.*, 2021)
Zebrafish	Injection (RNA)	Injection (RNA)	YTTN	35	21	90%	(Wierson *et al.*, 2019)

[a] *PGK1* promoter from *S. cerevisiae*, [b] *TDH3* promoter from *S. cerevisiae*, [c] *TEF1* promoter from *Aspergillus nidulans*, [d] *U6* promoter from *Aspergillus oryzae*, [e] *Ubi-1* promoter from *Zea mays*

designed to alter a single nucleotide. For instance, targeting a chromosomal region with low GC content could be more difficult using *Spy*Cas9. Therefore, increased flexibility in PAM recognition sequences is desired, and this has been one of the driving forces both for studies aiming at altering Cas9 PAM specificity (Hu *et al.*, 2018) and the search for alternative CRISPR-Cas systems (Li *et al.*, 2015, Zetsche *et al.*, 2015, Edraki *et al.*, 2019).

Class 2 type V CRISPR-Cas systems are one such alternative. The first type V Cas protein characterized in yeast was Cas12a (originally Cpf1) and was shown to differ from type II proteins in significant ways (Zetsche *et al.*, 2015, Swiat *et al.*, 2017, Verwaal *et al.*, 2018). Cas12a proteins recognize T-rich PAM sequences (5'-TTTV-3' or more general 5'-YTTN-3'), conversely to Cas9. Cas12a cleavage occurs distal from the PAM sequence and makes staggered end cuts, while Cas9 cuts proximal to the PAM and generates blunt ends. Interestingly, its effector module requires neither RNase nor tracrRNA to process its crRNA. The Cas12a effector protein recognizes the direct repeats preceding spacers in the CRISPR array and self-processes them to produce mature, single molecule crRNAs.

One interesting Cas12a protein is *Er*Cas12a (also referred to as MAD7), isolated from an *Eubacterium rectale* specimen found in Madagascar (Inscripta Inc., Boulder, CO, https://www.inscripta.com/resources/literature/) (Gill *et al.*, 2018). In contrast to previously characterized Cas12a nucleases from *Acidaminococcus*, and *Francisella* species that share high amino acid sequence identity (>95%), *Er*Cas12 exhibits less than 45% of identity with the above-mentioned nucleases while keeping all features of class 2 type V-A endonucleases. Interestingly, next to its technological advantages, *Er*Cas12a is currently the only Cas nuclease with a free commercial research license, stimulating the development of this endonuclease as a method for genome editing. *Er*Cas12a editing has been evaluated in several organisms (Liu *et al.*, 2019, Wierson *et al.*, 2019, Liu *et al.*, 2020, Price *et al.*, 2020, Jarczynska *et al.*, 2021, Lin *et al.*, 2021, Rojek *et al.*, 2021, Zhang *et al.*, 2021, Vanegas *et al.*, 2023) (Table 3.1), but fundamental questions remain about the optimal method of expression, critical design elements, and multiplex editing. These design elements, including the expression system, direct repeat (DR) length, spacer length and PAM, can have considerable impact on editing efficiency (Swiat *et al.*, 2017) and a consensus design has not yet been reached. There is thus a need to establish the optimal design features for implementing *Er*Cas12a editing in *S. cerevisiae*.

The goal of the present study was to optimize the *Er*Cas12a-based genome editing in *S. cerevisiae*. To this end, we explored ways to improve the efficiency of genome editing by tuning the length of the direct repeats and of the spacers. We also evaluated approaches to express the crRNA array by testing different designs and consequently proposed design principles for application of *Er*Cas12a in *S. cerevisiae*. Furthermore, we applied these principles to investigate applicability of *Er*Cas12a to multiplexing strategies.

3.2. Materials and Methods

Strains and cultivation conditions

The yeast strains used in this study are shown in Table 3.2. Strains were grown on complex yeast extract-peptone-dextrose (YPD) medium, consisting of 20.0 g L^{-1} glucose, 20.0 g L^{-1} Bacto peptone and 10.0 g L^{-1} Bacto yeast extract or on synthetic medium (SMD) containing 5 g L-1 $(NH_4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1.0 mL L^{-1} trace element solution and was supplemented with 20 g L^{-1} glucose and 1.0 mL L^{-1} vitamin solution (Verduyn *et al.*, 1992). Synthetic medium with urea as nitrogen source (SMD-urea) contained 6.6 g L^{-1} K_2SO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1.0 mL L^{-1} trace element solution and was supplemented with 20 g L^{-1} glucose, 1.0 mL L^{-1} vitamin solution and 2.3 g L^{-1} $CO(NH_2)_2$. When required, medium was supplemented with G418 (200 mg L^{-1}) or hygromycin B (200 mg L^{-1}). Solid medium was obtained by addition of 20.0 g L^{-1} Bacto agar. *S. cerevisiae* strains were grown in an Innova 44R shaker (Eppendorf, Hamburg, Germany) at 30 °C and 200 rpm in 500 mL shake flasks containing 100 mL medium or on stationary plates containing solid medium. For plasmid propagation, *E. coli* XL1-Blue cells (New England Biolabs, Ipswich, MA) were grown in 15 mL Greiner tubes containing 5 mL lysogeny broth (LB) medium at 37 °C and 200 rpm in an Innova 4000 Incubator Shaker (Eppendorf) or on stationary plates containing solid LB medium supplemented with 20.0 g L^{-1} Bacto agar. When required, LB medium was supplemented with ampicillin (100 mg L^{-1}), kanamycin (50 mg L^{-1}), spectinomycin (100 mg L^{-1}) or chloramphenicol (25 mg L^{-1}). Plates used for selection of *E. coli* transformants were grown stationary overnight at 37 °C and *S. cerevisiae* transformants were grown at 30 °C for three days in a stationary incubator. *S. cerevisiae* and *E. coli* cultures were stocked as 1 mL aliquots in a -80 °C freezer after addition of 30% (v/v) glycerol.

Molecular biology techniques

Gibson assembly was performed using Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA) according to the supplier's instructions. Golden Gate assembly was done according to Lee *et al.* (2015) with 20 fmol of each fragment with BsaI or BsmBI enzymes (New England Biolabs) and T7 DNA ligase (New England Biolabs). PCR for diagnostic purposes was performed using DreamTaq PCR Master Mix (Thermo Fisher Scientific, Walthman, MA) according to supplier's instructions. Phusion® High-Fidelity DNA polymerase (Thermo Fisher Scientific) was used for fragment amplification for cloning purposes according to supplier's instructions. All primers (Table S1) were ordered at Sigma-Aldrich (St Louis, MO). Isolation of yeast genomic material for PCR verification was done with the LiAc-SDS protocol according to (Lõoke *et al.*, 2011). Plasmid isolation was performed using GeneJET Miniprep kit (Thermo Fisher Scientific) and PCR products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific), both according to the supplier's instructions. When required, PCR products

Table 3.2. Strains used in this study.

Strain	Relevant genotype	Reference
CEN.PK113-7D	*MATa MAL2-8c SUC2*	(Entian & Kotter, 2007)
IMX2600	*MATa MAL2-8c SUC2 Δcan1::Spycas9-natNT2*	(Van den Broek *et al.*, 2024)
IMX2713	*MATa MAL2-8c SUC2 Δcan1::Spycas9-natNT2 ΔX-2*::pPGK1-Ercas12a-tPHO5*	This study
IME795	*MATa MAL2-8c SUC2* pGGKd018	This study
IME796	*MATa MAL2-8c SUC2* pUDE1093	This study
IMK1049, colony 1	*MATa MAL2-8c SUC2 Δcan1::Spycas9-natNT2 ΔX-2*::pPGK1-Ercas12a-tPHO5 Δade2*	This study
IMK1050, colony 2	*MATa MAL2-8c SUC2 Δcan1::Spycas9-natNT2 ΔX-2*::pPGK1-Ercas12a-tPHO5 Δade2*	This study
IMX2898, colony 1	*MATa MAL2-8c SUC2 Δcan1::Spycas9-natNT2 ΔX-2*::pPGK1-Ercas12a-tPHO5 ΔXI-3*::crtE ΔYPRCτ3*::crtI ΔII-1*::crtYB*	This study
IMX2899, colony 2	*MATa MAL2-8c SUC2 Δcan1::Spycas9-natNT2 ΔX-2*::pPGK1-Ercas12a-tPHO5 ΔXI-3*::crtE ΔYPRCτ3*::crtI ΔII-1*::crtYB*	This study

* X-2 and XI-3 from Mikkelsen *et al.*, (2012), YPRCτ3 from Flagfeldt *et al.*, (2009) and II-1 from Babaei *et al.*, (2021)

were isolated from gel using Zymoclean Gel DNA Recovery Kits (Zymo Research, Irvine, CA) according to the supplier's instructions. Unless specified otherwise, plasmid sequencing was done with Sanger sequencing by Macrogen (Amsterdam, The Netherlands).

Plasmid construction

Construction of *Er*Cas12a-expressing plasmid

The *Ercas12a* gene sequence was retrieved from Inscripta Inc. (https://www.inscripta.com/products/mad7-nuclease/, consulted April 2020, WP_055225123.1). To optimize protein expression and nuclear localization, the sequence was codon-optimized for *S. cerevisiae* and the nuclear localization signal (NLS) SV40 (Kalderon *et al.*, 1984) was added to the C-terminus. The plasmid pUDE1093 (Addgene #204227) expressing the *Er*Cas12a CRISPR nuclease from the strong constitutive *pPGK1* promoter and *tPHO5* terminator was constructed by Golden Gate cloning with BsaI of pYTK011, pGGKp177 and pUD1171 (co*Sc-Ercas12a*) into the backbone of pGGKd018, replacing the BsaI-flanked GFP dropout with the *Ercas12a* expression cassette. Correct assembly of pUDE1093 was verified by colony PCR using the primers 10320 & 10325 and sequence-verified by next-generation sequencing at Plasmidsaurus (Eugene, OR; https://www.plasmidsaurus.com/).

Table 3.3. Plasmids used in this study.

Plasmid	Genotype	Reference
pUDR547	*ori bla hph* 2µm gRNA X-2 gRNA X-2	(Postma *et al.*, 2021)
pYTK011	*ori cat pPGK1*	(Lee *et al.*, 2015)
pGGKp177	*ori aph3' tPHO5*	This study
pUD1171	*ori cat coSc-NLSSV40-Ercas12a*	GeneArt™
pGGKd018	*ori aadA1 kanMXR* 2µm GFPdo	(Randazzo *et al.*, 2021)
pUD530	*ori aph3'* SHRB panARSopt SHRC	GeneArt™
pUD532	*ori aph3'* SHRI *ori bla* SHRA	GeneArt™
pMEL12	*ori bla* 2µm *hphNT1* gRNA-CAN1.Y	(Mans *et al.*, 2015)
pMEL13	*ori bla* 2µm *kanMX* gRNA-CAN1.Y	(Mans *et al.*, 2015)
pUD1190	*ori aph3' pTDH3*-HH-S.DR-GFPdo-S.DR-HDV-*tTDH3*	GeneArt™
pUD1191	*ori aph3' pTDH3*-HH-L.DR-GFPdo-L.DR-HDV-*tTDH3*	GeneArt™
pUD1194	ori aph3' *pSNR52*-S.DR-GFPdo-S.DR-*tSUP4*	GeneArt™
pUD1195	ori aph3' pSNR52-L.DR-GFPdo-L.DR-*tSUP4*	GeneArt™
pUDE1093	*ori aadA1 kanMX* 2µm *pPGK1-Ercas12a-tPHO5*	This study (Addgene #204227)
pUDE1111	*ori bla hph* 2µm ConLS-*pTDH3*-ymNeongreen-*tADH1*-ConR1	This study
pUDE1112	*ori bla hph* 2µm ConLS-*pTDH3*-ymScarletI-*tADH1*-ConR1	This study
pUD1248	*ori cat pPGK1-crtYB-tPGK1*	This study
pUD1249	*ori cat pHHF2-crtE-tADH1*	This study
pUD1250	*ori cat pTDH3-crtI-tTDH1*	This study
pUDP239	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-L.DR-GFPdo-L.DR-HDV-*tTDH3*	This study
pUDP240	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-S.DR-GFPdo-S.DR-HDV-*tTDH3*	This study
pUDP241	*ori bla kanMX* panARS^{opt} *pSNR52*-L.DR-GFPdo-L.DR-*tSUP4*	This study
pUDP242	*ori bla kanMX* panARS^{opt} *pSNR52*-S.DR-GFPdo-S.DR-*tSUP4*	This study
pUDP285	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-L.DR-21nt crRNA *ADE2*-L.DR-HDV-*tTDH3*	This study
pUDP286	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-L.DR-25nt crRNA *ADE2*-L.DR-HDV-*tTDH3*	This study
pUDP287	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-S.DR-21nt crRNA *ADE2*-S.DR-HDV-*tTDH3*	This study

Table 3.3. (continued)

Plasmid	Genotype	Reference
pUDP288	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-S.DR-25nt crRNA *ADE2*-S.DR-HDV-*tTDH3*	This study
pUDP289	*ori bla kanMX* panARS^{opt} *pSNR52*-L.DR-21nt crRNA *ADE2*-L.DR-*tSUP4*	This study
pUDP290	*ori bla kanMX* panARS^{opt} *pSNR52*-L.DR-25nt crRNA *ADE2*-L.DR-*tSUP4*	This study
pUDP291	*ori bla kanMX* panARS^{opt} *pSNR52*-S.DR-21nt crRNA *ADE2*-S.DR-*tSUP4*	This study
pUDP292	*ori bla kanMX* panARS^{opt} *pSNR52*-S.DR-25nt crRNA *ADE2*-S.DR-*tSUP4*	This study
pUDP295	*ori blaR kanMX* panARS^{opt} *pTDH3*-HH-S.DR-crRNA XI-3-S.DR-HDV-*tTDH3*	This study
pUDP296	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-S.DR-crRNA YPRCτ3-S.DR-HDV-*tTDH3*	This study
pUDP311	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-S.DR-crRNA II-1 -S.DR-HDV-*tTDH3*	This study
pUDP312	*ori bla kanMX* panARS^{opt} *pTDH3*-HH-S.DR-crRNA XI-3-S.DR-crRNA YPRCτ3-S.DR-HDV-*tTDH3*	This study
pUDP313	*ori bla kanMX* panARSopt *pTDH3*-HH-S.DR-crRNA XI-3-S.DR-crRNA YPRCτ3-S.DR-crRNA II-1-S.DR-HDV-*tTDH3*	This study
pUDP293	*ori blaR hph* panARS^{opt} *pTDH3*-HH-S.DR-GFPdo-S.DR-HDV- *tTDH3, pPGK1-Ercas12a-tPHO5*	This study (Addgene # 204228)
pUDP299	*ori bla hph* panARS^{opt} *pTDH3*-HH-S.DR-crRNA *ADE2*-S.DR-HDV-*tTDH3, pPGK1-Ercas12a-tPHO5*	This study

Construction of *Er*Cas12a genome editing platform plasmids

The platform plasmids pUDP239-pUDP242 consisting of four parts were assembled via Gibson Assembly using unique 60 bp synthetic homologous recombination sequences (SHR-sequences A, F, C and I) incorporated during PCR amplification (Kuijpers *et al.*, 2013). The yeast marker cassette, A-*pAgTEF1-KanMX-tAgTEF1*-B, was amplified from plasmid pMEL13 (Mans *et al.*, 2015) using primers 3748 & 3749. The yeast origin B-panARS^{opt}-C was amplified from pUD530 (Gorter de Vries *et al.*, 2017) using primers 4672 & 3856. The *E. coli* origin and marker, I-*ori blaR*-A, was amplified from pUD532 (Gorter de Vries *et al.*, 2017) with primers 3274 & 3275. The gRNA insertion fragment was designed in four different combinations of promoter/terminator pair and DR sequences. The two DR sequences differ in length: a long DR of 35 nt (GTCAAAAGACCTTTTTAATTTCTACTCTTGTAGAT) and a short DR of 19 nt (AATTTCTACTCTTGTAGAT). Two types of promoter/terminator pairs were used: transcription by RNA polymerase III (*pSNR52* and tSUP4) or II in combination with ribozymes (*pTDH3* and *tTDH3*; hammerhead (HH) and hepatitis delta virus (HDV) ribozymes). Therefore, four different crRNA expression cassettes

were ordered (pUD1190, pUD1191, pUD1194 and pUD1195) from GeneArt (Thermo Fisher Scientific), each containing a GFP dropout site flanked with BsaI recognition sites for replacement by the crRNA for easy cloning. The plasmids were PCR-amplified with primers 3283 & 4068 to obtain the four different SHR-flanked crRNA insertion sites fragments with either a long or short DR and either a RNA polymerase II (*pTDH3*) or III (*pSNR52*) promoter. The PCR-amplified fragments were purified, and different platform plasmids were assembled by combining the appropriate fragments in a Gibson Assembly reaction. Correct assembly was verified by colony PCR of green colonies over the A, B, C and I SHR-sequences of pUDP239-pUDP242 using the primers 16503 & 8401, 9719 & 10345, 10344 & 4369 and 16501 & 6818, respectively.

The episomal plasmid enabling the expression of *Ercas12a* together with the crRNA was constructed by Gibson assembly of five fragments flanked with compatible SHR sequences (Kuijpers *et al.*, 2013). The yeast marker cassette A-*pAgTEF1-hphNT1-tAgTEF1*-B was amplified from plasmid pMEL12 (Mans *et al.*, 2015) using primers 3748 & 3749. The yeast origin F-panARS^{opt}-C was amplified from pUD530 (Gorter de Vries *et al.*, 2017) using primers 4672 & 3856. The *E. coli* origin and marker, I-*ori blaR*-A, was amplified from pUD532 (Gorter de Vries *et al.*, 2017) with primers 3274 & 3275. The crRNA insertion expression cassette (short DR and RNApolII design) was amplified from pUD1190 with primers 3283 & 4068. The *Ercas12a* expression cassette was amplified from pUDE1093 with primers 17934 & 9393. Gibson assembly of these five fragments resulted in pUDP293 (Addgene #204228). Correct construction was PCR-verified with primers 9719, 7487, 4377, 10345, 10344, 4369, 16501, 6818, 16503 and 8401. Also, the plasmid was Sanger sequenced using primers 3847, 3276, 4672, 7487, 7488, 19481, 19482, 19483, 19484, 19485, 19486, 3288, 3274 and 3275 and sequence-verified by next-generation sequencing at Plasmidsaurus (Eugene, OR).

General construction of crRNA expressing plasmids

The plasmids used in this study for crRNA expression were constructed according to a generalized Golden Gate cloning scheme (Figure 3.1). Platform plasmids contained a GFP dropout (GFPdo) within a crRNA expression cassette flanked by BsaI restriction sites, DRs, ribozymes in case of RNA polymerase II promoter/terminator regulation, and finally a promoter and terminator. Spacers were ordered as primer pairs with each primer containing four nucleotide overhangs complementary to the 5' or 3' four-nucleotide overhangs generated after BsaI restriction of the platform plasmid. The annealing of the primer pair thus creates a DNA fragment with four-nucleotide overhangs complementary to the restricted platform plasmid (Figure 3.1). Plasmids expressing crRNAs were constructed by Golden Gate assembly of a platform plasmid and a fragment of two annealed oligonucleotides, followed by green/white screening. Correct assembly of the crRNA into the platform plasmids was verified by diagnostic PCR and Sanger sequencing.

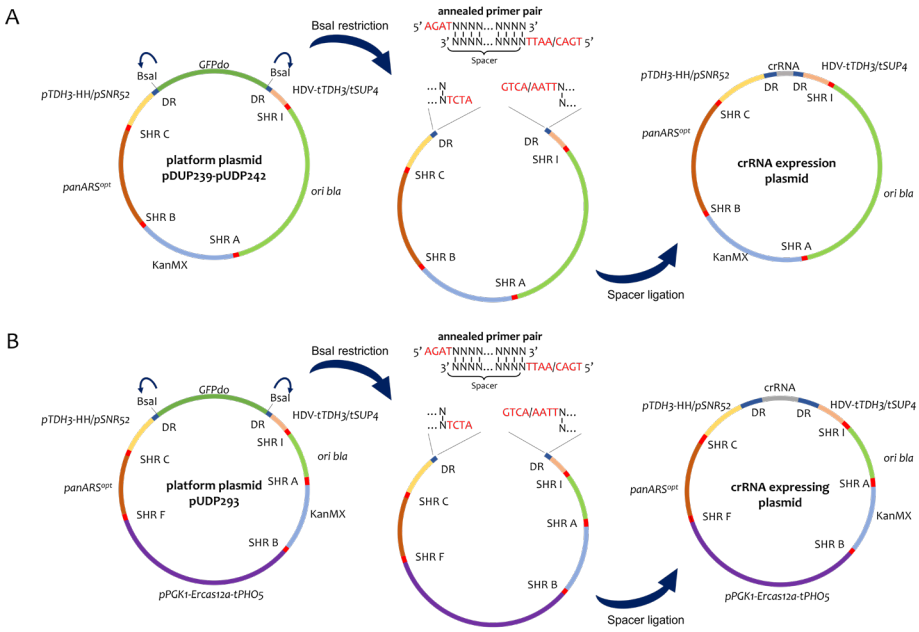


Figure 3.1. Construction of crRNA expression plasmids. Platform plasmids **A)** pUDP239-pUDP242 and **B)** pUDP293 carrying a crRNA expression cassette (promoter: yellow; DR: dark blue; terminator: pink) with GFPdo (dark green), *ColE1 ori bla* (light green), G418 yeast resistance marker cassette (*KanMX*) (blue), *pPGK1-Ercas12a-tPHO5* expression cassette (purple; **B**) and panARS^{opt} yeast origin of replication (Liachko & Dunham, 2014) (brown), separated by synthetic homologous recombination (SHR) sequences (red) (Kuijpers *et al.*, 2013). BsaI restriction is used to cut the plasmid at BsaI restriction sites. Sticky ends left from BsaI restriction are complementary to four-nucleotide overhangs of annealed spacer primer pair, which can also constitute a spacer-[DR-spacer]$_N$ array for multiplex editing at N+1 genomic targets. The 3' primer overhang sequence is dependent on the choice of DR. The oligo pair is annealed and ligated into the crRNA expression cassette, which generates the crRNA expression plasmid.

Construction of *ADE2* targeting plasmids and repair fragments

To construct crRNA-expressing plasmids targeting the *ADE2* gene in *S. cerevisiae*, complementary primers (18327, 18328, 18329, 19010, 19011 and 19012), containing a crRNA sequence targeting *ADE2*, were annealed and cloned into plasmids pUDP239, pUDP240, pUDP241 and pUDP242 via Golden Gate assembly with BsaI, resulting in plasmids pUDP285, pUDP286, pUDP287, pUDP288, pUDP289, pUDP290, pUDP291 and pUDP292, respectively. Inserts were PCR-verified with primers 10344 & 6097 and Sanger sequenced using primers 3283 & 11551. Repair fragments for *ADE2* deletion were constructed by annealing the primers 10155 & 10156.

Construction of XI-3, YPRCτ3, and II-1 crRNA expression plasmids and repair fragments

Plasmids expressing crRNA targeting the XI-3 (Mikkelsen *et al.*, 2012), YPRCτ3 (Flagfeldt *et al.*, 2009) and II-1 (Babaei *et al.*, 2021) loci individually, a duplex editing plasmid targeting XI-3 and YPRCτ3 and a plasmid targeting all three loci simultaneously using a multiplex crRNA array were constructed with platform plasmid pUDP240 as backbone. These three chromosomal locations were chosen as they are non-essential, thereby not interfering with any cellular activities, and promote high transcriptional activities. To construct crRNA-expressing plasmids pUDP295 (XI-3), pUDP296 (YPRCτ3), pUDP311 (II-1), pUDP312 (XI-3 and YPRCτ3) and pUDP313 (XI-3, YPRCτ3, and II-1), annealed primer pairs (primers 19489 & 19490 for XI-3, 19870 & 19871 for II-1, 19491 & 19492 for YPRCτ3, 19771 & 19772 for XI-3 and YPRCτ3 duplex editing and 19881 & 19882 for XI-3, YPRCτ3 and II-1 triplex editing) were cloned into pUDP240 via BsaI Golden Gate assembly. Correct construction was PCR-verified with primers 2655 & 6097 and Sanger sequenced using primers 2655 & 6097.

To construct repair fragments for single editing, pUDE1111 (*ori bla hphNT1* 2µm ConLS-*pTDH3*-ymNeongreen-*tADH1*-ConR1) was PCR-amplified with primers containing 60-bp overhangs homologous to the target: 15396 & 15397 for XI-3; 14022 & 14023 for YPRCτ3; 19773 & 19774 for II-1. For duplex editing, YPRCτ3 was repaired with the ymNeongreen fragment amplified from pUDE1111 with primers 14022 & 14023 and XI-3 was repaired with the ymScarletI fragment amplified from pUDE1112 (*ori bla hphNT1* 2µm ConLS-*pTDH3*-ymScarletI-*tADH1*-ConR1) with primers 15396 & 15397. For multiplex editing, *crtYB*, *crtE* and *crtI* transcriptional units were PCR amplified from pUD1248, pUD1249 and pUD1250 with primers 19883 & 19884, 19547 & 19548 and 19549 & 19550, respectively, and were used as repair fragments.

Construction of *Ercas12a*-expressing strains

A *S. cerevisiae* strain expressing the genes encoding the CRISPR nucleases *Ercas12a* and *Spycas9*, was constructed from the *Spycas9*-expressing strain IMX2600 (CEN.PK113-7D Δ*can1*::*Spycas9-natNT2*) (van den Broek *et al.*, 2024) (Table 3.2) using the CRISPR-Cas9-system (Mans *et al.*, 2015). The *Ercas12a* expression cassette was amplified from pUDE1093 using primers 11074 & 13597, incorporating flanks with homology to the X-2 genomic location (Mikkelsen *et al.*, 2012) required for *in vivo* HDR. IMX2600 was co-transformed with 500 ng of the plasmid pUDR547 expressing the gRNA targeting the X-2 intergenic region and 1000 ng of the *pPGK1-Ercas12a-tADH1* repair fragment. Transformants were selected on YPD plates supplemented with hygromycin B. Correct integration was verified by diagnostic PCR using the primers 13662 & 13663. After plasmid removal, the *Ercas12a*-expressing strain was named as IMX2713 (CEN.PK113-7D Δ*can1*::*Spycas9-natNT2*, ΔX-2::*Ercas12a*).

Genome editing procedure

crRNA design

All oligonucleotides targeting genomic sites were designed as two annealing primer sequences containing four-nucleotide overhangs complementary to the BsaI restriction overhangs of the plasmids. To design the spacer sequence, the CHOPCHOP tool (Labun *et al.*, 2019) was used to find a list of candidate spacers ranked by predicted functionality, searching for 5'-TTTV-3' PAM. This list was checked for RNA secondary structure with the RNAfold online tool (Lorenz *et al.*, 2011). Spacer sequences with no predicted secondary structure interfering with the DR sequence were checked for off-target effects with an in-house BLAST search tool against the IMX2600 strain genome and the top result with no off-targets was chosen.

Transformation procedure

For genomic editing, the recipient yeast strain was transformed in triplicate with 500 ng crRNA expression plasmid DNA and 1000 ng repair fragment DNA. In all cases, a control culture of the same strain was transformed with 500 ng of the crRNA expression plasmid without repair fragment. *S. cerevisiae* strains were transformed according to the LiAc/ssDNA/PEG transformation protocol (Gietz *et al.*, 1992). After transformation, 1 mL YPD was added to the cells for a 2 hour recovery at 30 °C prior to plating on selective medium. All transformations for determining editing efficiencies were performed in triplicate.

Genomic editing and screening

In all cases, transformants were genotyped by diagnostic PCR with primers specific for the targeted genomic location. In cases where *ADE2* was targeted, visual red/white screening was performed and up to ten red colonies and two white colonies were chosen for genotyping. In cases where the repair fragment contained an ymNeongreen expression cassette, transformants were visually screened under blue light for fluorescence before genotyping. Visual orange/white screening was used before PCR analysis for multiplex editing as the repair fragments each carried a different gene in the *Xanthophyllomyces dendrorhous* β-carotene pathway. Correct integration of all three genes leads to orange phenotype due to β-carotene accumulation (Verwaal *et al.*, 2018). Editing efficiency was calculated as colonies showing appropriate reporter phenotype (red/fluorescent/orange) over total transformants.

Growth rate determination

To determine maximum specific growth rates on glucose, the yeast strains were cultivated on SMD. When selective pressure for plasmid maintenance was required, IME795 (CEN.PK113-7D pGGKd018) and IME796 (CEN.PK113-7D pUDE1093) were

grown on SMD-urea supplemented with G418. Cultures were inoculated from stocked aliquots, grown overnight at 30 °C and transferred to fresh medium. From this pre-culture, the experimental cultures were inoculated in triplicate at an OD660 of 0.2 on their corresponding medium. Optical density (OD) was measured at set time intervals with a Jenway 7200 scanning spectrophotometer (Cole-Parmer Inc, Chicago, MI) at 660 nm. To remove plasmids from IME795 and IME796, strains were restreaked on nonselective medium, until restreaking on selective medium (G418) yielded no colonies. Maximum specific growth rates were determined by plotting the natural logarithm of the growth curve and performing linear regression on the exponential portion of the growth curve (at least five data points).

Analytical techniques

Supernatant samples of liquid cultures were collected and glucose and ethanol concentrations were analysed on an Agilent 1260 Infinity HPLC (Agilent Technologies Inc, Santa Clara, CA) equipped with an Animex HPX-87H ion exchange column (Bio-Rad, Hercules, CA) at 60 °C and 5 mM H_2SO_4 solution as mobile phase at a flow rate of 0.6 mL min^{-1}.

Flow cytometry and cell sorting

After the transformation procedure, the cell suspension was plated onto selective medium (1/5 of the cells) and transferred to 20 mL liquid selective medium in 100 mL shake flasks (4/5 of the cells) and cultivated for 2 days at 30 °C while shaking at 200 rpm. Then, 1 mL of the cell suspension was transferred to 20 mL fresh YPD medium supplemented with 200 mg L^{-1} of hygromycin in 100 mL shake flasks and grown for 2 days. Finally, 1 mL cell suspension was transferred to 100 mL non-selective YPD medium in 500 mL shake flasks and grown for 1 day at 30 °C while shaking at 200 rpm. *In vivo* assembly efficiency of ymNeongreen and ymScarletI genes in IMX2713 was analyzed by measuring fluorescence levels in the BD FACSAria™ II SORP Cell Sorter (BD Biosciences, Erembodegem-Dorp, Belgium) equipped with 355, 445, 488, 561 and 640 nm lasers and a 70 µm nozzle and operated with FACSFlow™ software (BD Biosciences). The fluorophore ymScarletI was excited by the 561 nm laser and emission was detected through a 582 nm bandpass filter with a bandwidth of 15 nm. The fluorophore ymNeongreen was excited by the 488 nm laser and emission was detected through a 545 nm bandpass filter with a bandwidth of 30 nm. The cytometer performance was evaluated prior to each experiment by running a CST cycle with CS&T Beads (BD Biosciences) and the drop delay for sorting was determined by running an Auto Drop Delay cycle with Accudrop Beads (BD Biosciences). For each sample, 100.000 events were analyzed. Cell morphology was analyzed by plotting forward scatter (FSC) against side scatter (SSC) and the appropriate cell size was gated. Gated cells were used to determine the fluorescence intensity of the cells. Gating windows for fluorescence intensity were based on the fluorescence of the cells transformed with

solely ymNeongreen or ymScarletI as repair fragment for sole integrations. Cells in the gate ymNeongreen^{+}ymScarletI^{+} were sorted separately on non-selective YPD plates and grown for 2 days. FACS data was analysed using the Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Finland).

Whole genome sequencing

Yeast genomic DNA of transformants IMX2713, IMK1050, IMK1051, IMX2898 and IMX2899 was isolated using QIAGEN Genomic-tip 100/G kit (Qiagen, Hilden, Germany), following manufacturer's instructions. Genomic DNA concentrations were measured with the BR dsDNA kit (Invitrogen, Carlsbad, CA) using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Whole genome sequencing using TruSeq DNA PCR-Free Library Preparation (150 bp paired ends, 350 bp insert) on a NovaSeq 6000 S4 sequencer was performed by Macrogen-Europe (Amsterdam, The Netherlands).

All Illumina sequencing (Table S5) data are available at NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject accession number PRJNA977855) . The raw Illumina reads were mapped using the Burrows–Wheeler Alignment tool (BWA) (Li, 2013) against a chromosome-level reference genome of IMX2600 (NCBI bioproject accession number PRJNA976676) (van den Broek *et al.*, 2024) to which four extra contigs containing the *Ercas12a* and *Xdcrt* integration cassettes were added (Supplementary Sequences). The alignments were further processed with SAMtools (Danecek *et al.*, 2021) and visualized using the Integrative Genomics Viewer (IGV) (Robinson *et al.*, 2011). Sequence variants were called using Pilon (Walker *et al.*, 2014), ReduceVCF (https://github.com/AbeelLab/genometools/blob/master/scala/abeel/genometools/reducevcf/ReduceVCF.scala) was used to extract the variants and VCFannotator (http://vcfannotator.sourceforge.net/) was used to annotate the variants.

3.3. Results

Construction of a *Er*Cas12a nuclease expressing *S. cerevisiae* strain

To evaluate *Er*Cas12a-mediated genome editing in *S. cerevisiae*, two strategies for nuclease expression were explored. Expression from a chromosomal location ensures stable gene expression, allows recurrent use of the endonuclease and a constant selective pressure is not required, whereas plasmid-based expression allows for quick removal of the nuclease-encoding DNA after the editing event is completed and enables easy transfer into different yeast lineages. Plasmid-free nuclease expression requires integration of the respective expression cassette into the genome of *Saccharomyces cerevisiae*. First, the *cas12a* gene from *Eubacterium rectale* (*Ercas12a*) was codon-optimized for *S. cerevisiae* and the SV40 nuclear localization sequence was added to the C-terminus of the protein to ensure nuclear localization. The *Ercas12a* gene was

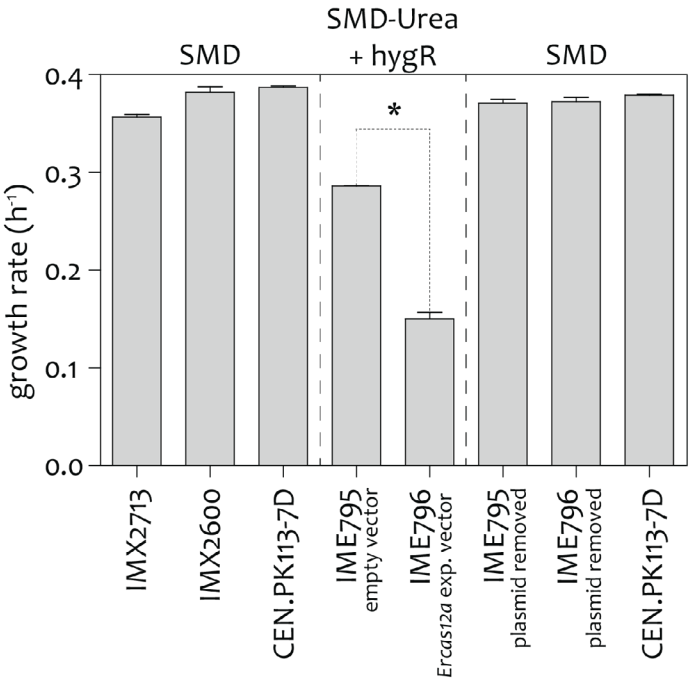


Figure 3.2. Maximum specific growth rate of strains expressing *Er*Cas12a and their control strains. IMX2713 expressing *Er*Cas12a from its genomic DNA, control strain IMX2600 and CEN.PK113-7D were cultivated in shake flasks on synthetic medium with glucose as sole carbon source. IME796 expressing ErCas12a from the multicopy plasmid pUDE1093 (2µ *KanMX pTDH3*-GFPdo-*tADH1 pPGK1-Ercas12a-tPHO5* and its reference strain IME795 containing the empty vector pGGKd018 (2µm *KanMX*) were grown on synthetic medium with glucose as carbon source and urea as nitrogen source supplemented with G418 for selective pressure for plasmid maintenance. After plasmid removal, the strains were grown on synthetic medium with glucose as carbon source with CEN.PK113-7D as control strain. The measurements were performed in two (IMX2713 and IMX2600) or three (IME795 & IME796) biological replicates and two technical replicates per sample. Significant differences in specific growth rate relative to the control strain based on a two-tailed homoscedastic paired t-test are indicated with * ($p<0.001$).

cloned under control of the constitutive *PGK1* promoter and the *PHO5* terminator. The *Ercas12a* expression cassette was integrated into the X-2 site (Mikkelsen *et al.*, 2012), regarded as safe integration site, using the *Spycas9*-system (Mans *et al.*, 2015), guided by a specific gRNA expressed from pUDR547, in the *S. cerevisiae* strain IMX2600 (CEN.PK113-7D Δ*can1*::*Spycas9-natNT2*), resulting in IMX2713 (CEN.PK113-7D Δ*can1*::*Spycas9-natNT2* ΔX-2::*Ercas12a*). As Cas9 and Cas12a endonucleases use different crRNA structures (Cas9 relies on the structural part corresponding to the tracrRNA, whereas Cas12a recognizes direct repeat sequences) and require the presence of different PAM sequences (5'-NGG-3' for Cas9 and 5'-TTTV-3' for Cas12a), these were hypothesized to not interfere while present simultaneously.

To study the impact of *Ercas12a* expression in IMX2713, the growth rate on synthetic medium with glucose as sole carbon source was determined. The strain IMX2713 (CEN.PK113-7D *Δcan1::Spycas9-natNT2* ΔX-2::*Ercas12a*) exhibited a non-significant 10% decrease in growth rate (0.36 ± 0.004 h^{-1}) in comparison to its parental strain IMX2600 (0.39 ± 0.010 h^{-1}) and laboratory strain CEN.PK113–7D (0.40 ± 0.001 h^{-1}), demonstrating that genomic integration of *Ercas12a* had no significant impact on growth (Figure 3.2).

To evaluate whether increased expression negatively impacts growth of *S. cerevisiae*, *Ercas12a* was expressed from the 2μm (high copy) plasmid pUDE1093 under control of the same constitutive promoter used in IMX2713. The strain IME796 (pUDE1093 (*pPGK1-Ercas12a-tPHO5*)) grew significantly slower with a 52% reduction of the maximum specific growth rate relative to the control strain IME795 that harboured the pGGKd018 empty vector ($IME796_{\mu max}$ = 0.14 ± 0.00 h^{-1}, $IME795_{\mu max}$ = 29 ± 0.00 h^{-1}). We hypothesized that an increased gene copy number of the plasmid-borne expression system might result in enhanced gene expression of *Er*Cas12a, that in return could be toxic to the cell (Figure 3.2). Curing the plasmids fully restored specific growth rate in strains IME796-pUDE1093 and IME795-pGGKd018 to that of the CEN.PK113-7D reference strain. On SMD with glucose as carbon source, the reference strain CEN.PK113-7D grew with a growth rate of 0.379 ± 0.00 h^{-1}, IME796-pUDE1093 and IME795-pGGKd018 grew with a growth rate of 0.371 ± 0.01 h^{-1} and 0.376 ± 0.00 h^{-1}, respectively (Figure 3.2). These results were in line with earlier characterization of *Fn*Cas12a and *Spy*Cas9, where plasmid-borne expression of CRISPR endonucleases led to similar toxic effects and reduction of growth rates (Generoso *et al.*, 2016, Swiat *et al.*, 2017).

Defining parameters for optimal *Er*Cas12a-mediated genome editing in *S. cerevisiae*

To optimize the editing procedure with *Er*Cas12a in *S. cerevisiae*, a series of constructs with variable designs for the crRNA expression cassette were made to evaluate the effect of different promoter systems, length of direct repeats and spacer length on editing efficiency. Two expression cassettes of the crRNA were assessed, the first was based on the RNA polymerase III (RNApolIII) regulatory sequences *pSNR52* and *tSUP4*, classically used to express crRNA; and the second was based on the RNA polymerase II (RNApolII)-dependent *TDH3* regulatory sequences *pTDH3*/*tTDH3*. Since RNA polymerase II expression concomitantly results in 5' RNA capping and 3' poly-A tailing, the *pTDH3*/*tTDH3* constructs incorporated both HammerHead (HH) and Hepatitis Delta Virus (HDV) ribozymes enabling maturation of the crRNA after self-cleavage (Gao & Zhao, 2014). Two DR sequences were tested with either the short 19-nt 5'-AATTTCTACTCTTGTAGAT-3' or the longer 35-nt 5'-GTCAAAAGACCTTTTTAATTTCTACTCTTGTAGAT-3' DR. The long DR corresponds to the DR found in the *E. rectale* CRISPR array was used in most of the studies implicating *Er*Cas12a (Table 3.1). A previous study established that editing in *S. cerevisiae* using the Cas12a nuclease from *Francisella novicida* (*Fn*Cas12a) was more efficient when using shorter DRs corresponding to the highly conserved core

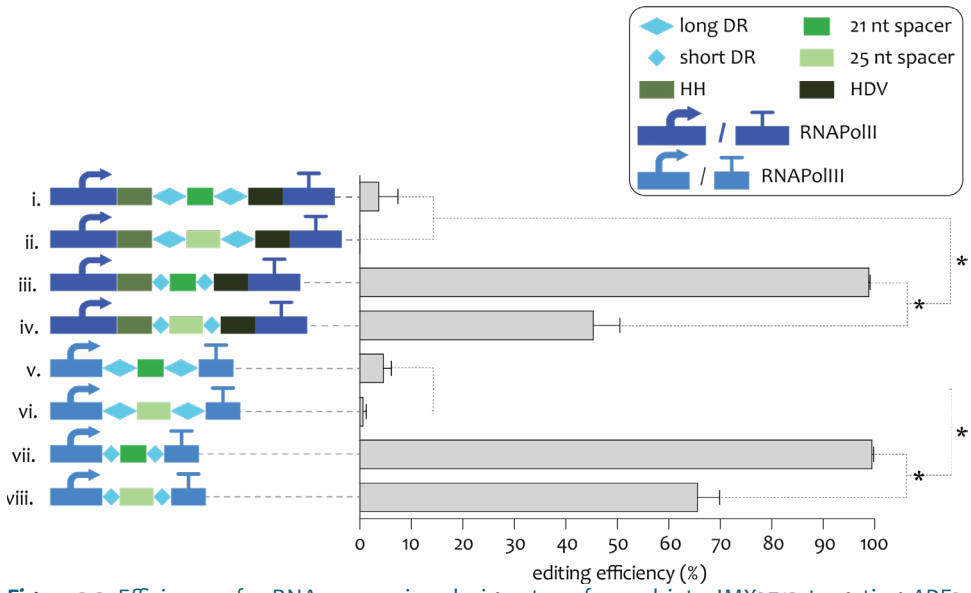


Figure 3.3. Efficiency of crRNA expression designs transformed into IMX2713 targeting *ADE2*. Different combinations of expression system, spacer length and DR length were investigated: **i-iv)** RNApolII (*pTDH3* and *tADH1*) expression system and ribozymes flanking the DR-spacer-DR sequences; **v-viii)** RNApolIII (*pSNR52* and *tSUP4*) expression system; **i,ii,v,vi)** long (35 nt) DR sequences; **ii, iv, vi, viii)** short (19 nt) DR sequences; **i,iii,v,vii)** short (21 nt) spacer; **ii,iv,vi,viii)** long (25 nt) spacer. Efficiency was calculated as the ratio of red transformants over the total number of transformants of three biological replicates. Significant differences in editing efficiencies based on a two-tailed homoscedastic paired t-test are indicated with a * ($p<0.001$).

region of the Cas12a DR among prokaryotes, than when using the native long DR (Swiat *et al.*, 2017, Randazzo *et al.*, 2021). Additionally, two spacers of 21 nt or 25 nt were evaluated. All eight combinations (23) were constructed and combined using spacer sequences that target the *S. cerevisiae ADE2* locus; deletion of *ADE2* causes an accumulation of 5-amino-imidazole ribonucleotide that upon oxidation produces a red/pink colour (Dorfman, 1969), facilitating the visual detection of the edited transformants (Figure 3.3). The spacers CCGGTTGTGGTATATTTGGTGTGGA (25 nt) and CCGGTTGTGGTATATTTGGTG (21 nt) were selected based on the presence of a 5'-TTTV-3' PAM sequence located at T738 within the *ADE2* open reading frame. Separately, the eight plasmids targeting *ADE2* (pUDP285-pUDP292), were co-transformed with a 120 bp repair oligo containing 60-bp homology upstream and downstream the *ADE2* gene for proper chromosomal repair using the native yeast HDR machinery into the *S. cerevisiae* strain IMX2713 (*can1Δ::Spycas9-natNT2 X-2Δ::pPGK1-Ercas12a-tPHO5*).

For each transformation, ten red and two white colonies were genotyped by diagnostic PCR to confirm the correct *ADE2* deletion (Figure S1). Since all genotyped red colonies harboured the expected deletion and the white colonies still showed presence of the wild-type *ADE2* allele, the targeting efficiencies (%) were estimated by the ratio of red

colonies over all transformants. The most impactful design element was the length of the DR, as constructs carrying the long DR (35 nt) did not exceed a targeting efficiency of 5%. This is remarkable, since the long DR sequence for *Er*Cas12a has been widely used in previous literature (Table 3.1). In contrast, all designs that included the short DR version (19-nt) exhibited at least 45% targeting efficiencies regardless of the spacer length and the promoter systems used (Figure 3.3, Table S2). Next, from the four configurations with short DRs, the two constructs with shorter spacers (21 nt) were significantly better, reaching over 99% efficiency irrespective of the expression system used. As the strain IMX2713 expresses both the *Er*Cas12a and *Spy*Cas9 endonucleases, the strain IMX2600 solely expressing Cas9 was transformed with the pUDP287 plasmid targeting *ADE2* with the highest efficiency and its repair fragment. The absence of red colonies and high number of white colonies shows that Cas9 and crRNAs designed for Cas12a are incompatible with each other and the observed editing is purely performed by *Er*Cas12a (Table S3).

Overall, these data indicated that a combination of 21 nt spacer and short flanking DR represented an optimized design for highly efficient *Er*Cas12a-mediated genome editing in *S. cerevisiae*. Due to the applicability of RNApolII expression systems across different *Saccharomycotina* yeast species, it was decided to continue with the RNApolII design. Based on these considerations, pUDP240 (*pTDH3*-HH-S.DR-GFPdo-S.DR-HDV-*tTDH3*) containing a versatile GFP dropout construct for easy cloning of crRNAs was chosen as basic plasmid architecture for further experiments (Figure 3.1A).

*Er*Cas12a can edit commonly used integration sites with high efficiency in *S. cerevisiae*

For metabolic engineering applications, it is important that the engineered function (e.g. heterologous metabolic pathway) remains mitotically stable, which is usually achieved through chromosomal integration. To further assess the applicability of *Er*Cas12a to engineer the *S. cerevisiae* genome, we have selected three non-essential chromosomal regions that promote high transcriptional activities: XI-3 (Mikkelsen *et al.*, 2012), YPRCτ3 (Flagfeldt *et al.*, 2009) and II-1 (Babaei *et al.*, 2021). Conversely to *ADE2* editing, the successfully targeted transformants showed no distinctive phenotype. Therefore, to determine the targeting efficiency, a repair DNA fragment including the ymNeongreen fluorescent reporter gene (Botman *et al.*, 2019) flanked with corresponding homology arms was used. To determine the editing efficiency, the strain IMX2713 (*can1Δ::Spycas9-natNT2* X-2Δ::*pPGK1-Ercas12a-tPHO5*) was transformed with pUDP296 (crRNA XI-3), pUDP300 (crRNA YPRCτ3) and pUDP311 (crRNA II-1) and their respective repair fragments (Table S4). The transformations targeting the XI-3 and YPRCτ3 loci yielded a 100% editing efficiency as all transformants exhibited a fluorescent phenotype, resulting from the integration of the ymNeongreen cassette. The transformation targeting II-1 reached a near 100% editing efficiency of 99.1 ± 0.8%. Similarly to *ADE2*, transforming IMX2600 with the *Er*Cas12a-designed crRNAs targeting

XI-3, YPRCτ3 and II-1 did not result in editing (Table S3). As precedingly performed for *ADE2*, for each transformation, ten green fluorescent colonies were subjected to genotyping and all fluorescent transformants were shown to have a correct insertion of ymNeongreen repair at the targeted genomic site (Figure S2).

*Er*Cas12a can process multispacer crRNA arrays for multiplex editing in *S. cerevisiae*

The most attractive feature of class 2 type V-A nucleases over class 2 type II is their ribonuclease activity, enabling self-processing of the pre-crRNA array. This property becomes pertinent when several crRNAs have to be expressed simultaneously to target different chromosomal locations in a so-called multiplexing editing approach. By combining multiple spacers interspaced by DR sequences in a single array, the Cas12a nuclease can cleave the polycistronic pre-crRNA array into single mature crRNAs (Figure 3.4A). To assess the multiplexing ability of *Er*Cas12a in *S. cerevisiae*, the previously tested spacers targeting XI-3 and YPRCτ3 were combined in a single DR-spacer-DR-spacer-DR array and cloned into pUDP240 yielding pUDP312 (Table 3.3). The use of repair fragments expressing the fluorescent proteins ymScarletI and ymNeongreen for resolving the *Er*Cas12a-induced DSB at XI-3 and YPRCτ3, respectively, enabled analysis of the transformed population by flow cytometry (Figure 3.4B). A 96.5 ± 4.9% editing efficiency was obtained for duplex editing of the XI-3 and YPRCτ3 genomic integration sites (Figure 3.4C, Figure S3, Table S4). These results showed that the *Er*Cas12a nuclease can process a polycistronic crRNA array for multiplex editing.

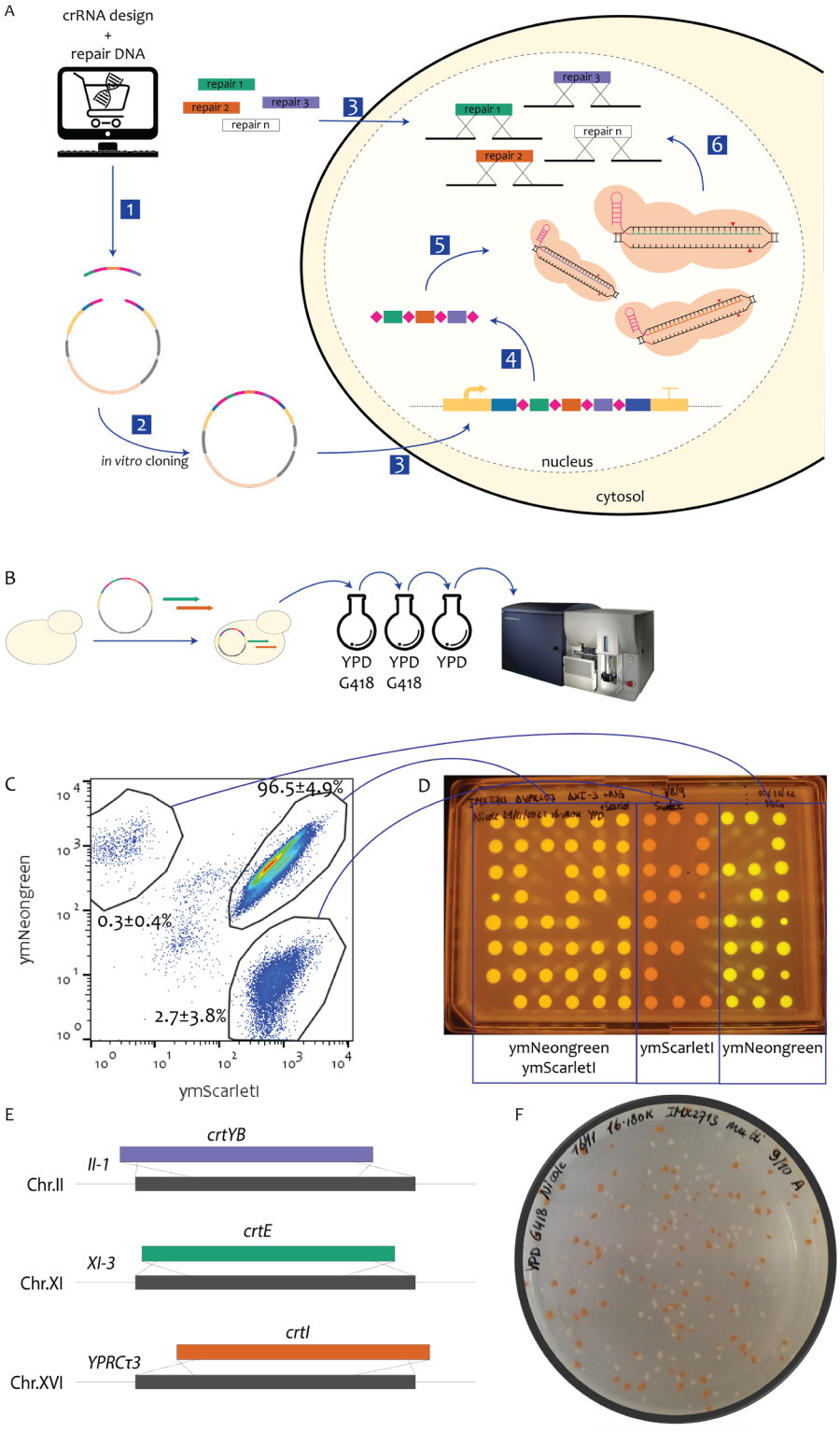
Similarly, a polycistronic crRNA array comprising the previously tested spacers targeting XI-3, YPRCτ3 and II-1 were combined in a single array and cloned into pUDP240 yielding pUDP313 (Table 3.3). The repair of the three DSBs introduced at XI-3, YPRCτ3 and II-1 was achieved by co-transforming three repair DNA fragments containing respectively the *Xanthophyllomyces dendrorhous* carotenogenic genes *crtE*, *crtI* and *crtYB* (Figure 3.4D). Simultaneous expression of the three *Xdcrt* genes in *S. cerevisiae* leads to accumulation of β-carotene which colour the yeast colonies orange (Verwaal *et al.*, 2007). Co-transformation of IMX2713 with the plasmid encoding the multispacer crRNA array and the β-carotene genes resulted in an editing efficiency of 31.3 +/- 6.6% (Figure 3.4E, Table S4). Whole genome sequencing of two orange colonies verified the integration of the three β-carotene genes into their respective genomic locations (Figure S4). Genotyping of ten white colonies revealed that in all cases, the integration of the *XdcrtYB* gene into the II-1 genomic location was missing and in two colonies the *XdcrtI* gene in YPRCτ3 as well, resulting in absence of β-carotene accumulation (Figure S5).

Whole genome sequencing of *Er*Cas12a-edited strains strongly suggests absence of off-targeting

Although the programmable specificity of Cas9 and Cas12a primarily depends on the

spacer sequence of the gRNA and the presence of a PAM sequence at the genomic target, off-target cleaving activity could still take place at sequences with up to five mismatches in the gRNA sequence (Zhang *et al.*, 2015). Therefore, potential off-target DSBs in CRISPR-mediated editing may pose a major concern in phenotypic analysis. To investigate the extent of off-targeting by *Er*Cas12a, two Δ*ade2* strains (IMK1050 and IMK1051) and two β-carotene-producing strains (IMX2898 and IMX2899), obtained by *Er*Cas12a genome editing, were subjected to whole genome sequence analysis (Table S5). Compared to the parental strain IMX2713 (*Spycas9 Ercas12a*), IMK1050 (*Spycas9 Ercas12a Δade2*) had one nucleotide variation (CHRXIII in gene *TRI1* C654A, resulting in Asn218Lys) and IMK1051 (*Spycas9 Ercas12a Δade2*) had four nucleotide variations in intergenic regions (CHRI A408C, CHRXI A465625T, CHRXIV A478C and CHRXV G637531C). Similarly, the analysis of the genome sequence of the carotenogenic *S. cerevisiae* strains IMX2898 and IMX2899 (*Spycas9 Ercas12a XdcrtE XdcrtI XdcrtYB*) did not reveal many single nucleotide variations (SNVs). IMX2898 harboured one SNV (CHRXI in gene *SSH4* G370C resulting in Glu124Gln) and IMX2899 four (CHRIII in YEL074W C122A causing Pro41His and the synonymous mutation A120C, CHRIV in *NUM1* T4188G causing Asn-1396-Lys and CHRXIV A478C in an intergenic region) of which one SNV located in telomeric region of CHRXIV was also observed in IMK1051 (Table S6). The detection of mutations A408C and A478C on CHRI and CHRXIV, which are both located in the telomeres of their respective chromosomes, stresses that these SNVs occur in rather redundant regions of the genome. Moreover, nucleotide BLAST analysis of the region of the SNV (± 500 bp upstream-downstream of the SNV location) did not identify a potential PAM (TTTV) and spacer seed sequence susceptible to off-targeting. Thus, there was no indication that the mutations in these strains were located near

Figure 3.4. A) Overview of multiplex genome editing in *S. cerevisiae* with *Er*Cas12a. **1.** crRNAs and repair DNA are designed and ordered as oligos. **2.** crRNAs are cloned into the crRNA expression plasmid using the pre-designed BsaI compatible overhangs. **3.** The *Er*Cas12a-expressing strain IMX2713 is transformed with the cRNA-expressing plasmid and the DNA repair fragments. **4.** The pre-crRNA multispacer array is transcribed and processed by the flanking ribozymes. **5.** *Er*Cas12a self-processes the pre-crRNA array into mature crRNAs, recognizes the pseudoknot and is guided to the target location. **6.** *Er*Cas12a induces precise DSBs and the native homology-directed repair machinery resolves the DSB using the supplied repair DNA fragment as template, thereby incorporating the intended genomic modification. **B)** Screening strategy for the evaluation of duplex editing in IMX2713. IMX2713 was co-transformed with pUDP312 and repair DNA fragments ymScarletI and ymNeongreen for targeting and integration in XI-3 and YPRCτ3 respectively. After transformation, the cells were cultivated on YPD G418 medium, transferred to fresh selective medium, transferred to non-selective YPD and screened by FACS. **C)** FACS screening of the transformed cell population. Cells were plotted for ymNeongreen (y-axis) and ymScarletI (x-axis) fluorescence levels and gated for single (ymNeongreen upper left gate, ymScarletI, bottom right gate) or double (ymNeongreen and ymScarletI, upper right gate) fluorescence. **D)** Sorted cell populations grown on YPD. **E)** Schematic representation of multiplex editing strategy for integration of *crtE*, *crtI* and *crtYB* in XI-3, YPRCτ3 and II-1, respectively. **F)** Plate of grown cells after co-transforming of IMX2713 with pUDP311 targeting XI-3, YPRCτ3 and II-1 and the respective DNA repair fragments encoding *crtE*, *crtI* and *crtYB*. Orange colonies indicate correct integration of all three *Xdcrt* genes due to accumulation of the orange pigment β-carotene.


A
crRNA design
+
repair DNA
repair 1
repair 2
repair 3
repair n
1
2
in vitro cloning
3
3
4
5
6
nucleus
cytosol
B
YPD
G418
YPD
G418
YPD
C
96.5±4.9%
0.3±0.4%
2.7±3.8%
ymNeongreen
ymScarletI
D
ymNeongreen
ymScarletI
ymScarletI
ymNeongreen
E
crtYB
Il-1
Chr.II
crtE
XI-3
Chr.XI
crtI
YPRCτ3
Chr.XVI
F

sequences recognizable by *Er*Cas12a based on the supplied crRNAs. Moreover, comparing the sequence of IMX2713 (*Ercas12a* and *Spycas9*) to IMX2600 (*Spycas9*) yielded limited mutations (one hit in the *MSH* gene located on CHRIV and eleven mutations in intergenic locations), thus solely expressing *Ercas12a* without presence of a crRNA spacer sequence does not result in off-targets. Since a connection between the mutations in the different strains was absent, these mutations were likely to have been introduced as a result of regular DNA replication during cell division rather than a consequence of genome editing with *Er*Cas12a.

Designing and testing a transportable *Er*Cas12a-mediated editing platform

To foster the use of this CRISPR tool, a plasmid combining the *Ercas12a* gene and the crRNA expression cassettes was constructed that can easily be transferred to other *S. cerevisiae* lineages without investing time in constructing a strain expressing the endonuclease from a chromosomal location. As demonstrated earlier, such overexpression of *Ercas12a* from a high copy number plasmid significantly reduced the growth rate. The transportable editing system pUDP293 (Figure 3.1B) was designed to express *Ercas12a* and contained the easy-to-clone crRNA insertion part with the previously determined optimal configurations for crRNA expression. The highly efficient *ADE2* spacer was cloned into the transportable plasmid to test the functionality of the transportable *Ercas12a* expressing plasmid and to determine the editing efficiency with episomally expressed *Ercas12a*. Therefore, the *S. cerevisiae* wild type strain CEN.PK113-7D was co-transformed with the transportable plasmid along with repair fragments for *ADE2* deletion. Based on the ratio of red colonies over the total number of transformants, an editing efficiency of 31.5 +/- 11.5% was determined (Table S4). Ten red colonies were subjected to genotyping for the *ADE2* deletion and confirmed the deletion of *ADE2* (Figure S6). The editing efficiency obtained with this plasmid-borne *Ercas12a* expression was threefold lower than with *Ercas12a* expression from a chromosomal locus. Although reduced, the efficacy of this plasmid system will facilitate the application of *Er*Cas12a allowing for genome editing in multiple *Saccharomyces* lineages and this editing system has also the potential to be transferred in other yeast species (Liachko & Dunham, 2014).

3.4. Discussion

The CRISPR-Cas genome editing technology has greatly accelerated strain engineering in the last decade. However, the use of the hallmark endonuclease *Spy*Cas9 is impeded by uncertainties such as a complex patent landscape (Ledford, 2022, Shaffer, 2022). In addition, although already widespread, the *Spy*Cas9 imposes design principles that sometimes might restrict its field of application, such as its GC-rich PAM sequence, a gRNA that includes a complex structural elements, which prevent implementation of simple multiplex editing design (Swarts & Jinek, 2018). Therefore, access to CRISPR

systems with clearer and less restrictive intellectual property rights and a more flexible multiplexing design would further promote democratization of the CRISPR editing technologies.

One alternative CRISPR-Cas system relies on the class 2 type V endonuclease *Er*Cas12a. In contrast to *Spy*Cas9, the *Er*Cas12a that was discovered by Inscripta Inc. (https://www.inscripta.com/) (Gill *et al.*, 2018) can be used for research and development and academic purposes through a permissive licensing agreement. Although *Er*Cas12a has been studied in various species (Liu *et al.*, 2019, Wierson *et al.*, 2019, Liu *et al.*, 2020, Price *et al.*, 2020, Jarczynska *et al.*, 2021, Lin *et al.*, 2021, Rojek *et al.*, 2021, Zhang *et al.*, 2021, Vanegas *et al.*, 2022) (Table 3.1) including *S. cerevisiae*, the targeting efficiency varied extremely per organism and greatly depends on the parameters used. Especially, the model microorganism *S. cerevisiae* only reached an editing efficiency of 66%, and the parameters were not reported (Inscripta Inc). Here, we demonstrated that efficiency of genome editing using the *Er*Cas12a system could be improved to reach nearly 100%, when the endonuclease is integrated in the genome. In addition, to go beyond the canonical CRISPR-Cas9, the *Er*Cas12a further expanded genome accessibility by facilitating access of AT-rich regions.

Our work confirmed and expanded design guiding principles for the construction of gRNA expression cassettes of Cas12a enzymes. Of all tested parameters, the length of the CRISPR direct repeat sequence was the most critical. Out of the two configurations tested, the use of the shorter DR (19-nt) systematically resulted in a better targeting efficiency (Figure 3.3). Similar results were reported for the application of the *F. novicida* Cas12a in *S. cerevisiae* (Swiat *et al.*, 2017), demonstrating that this was not specific to a single Cas12a enzyme, but that this principle could be potentially extended to all class 2 type V endonucleases. One attractive feature of Cas12a endonucleases is their RNase activity that allows the nuclease to self-process the crRNA. The result of this ribonuclease activity is a mature crRNA with a 19 nt DR (Zetsche *et al.*, 2017). The size reduction of the DR from 35 to 19 nt (Table 3.1) might allow skipping of the pre-crRNA self-processing steps into the mature crRNA, which would enhance the synthesis of the mature crRNA and therefore improve targeting efficiency. While the use of short DRs for *Fn*Cas12a has already been performed successfully in other living systems (Zetsche *et al.*, 2017), whether this holds true for *Er*Cas12a editing in other yeast species beyond *S. cerevisiae* requires further investigation. Next to the design of the DR structural part of the crRNA, the design of a spacer specific for the editing event is crucial and, based on the presented results, guidelines for crRNA design for *Er*Cas12a can be summarized as: i) find a 5'-TTTV-3' PAM sequence at the genomic targeted site of interest, ii) the GC-content of the spacer sequence can be between 30-70%, iii) add the 19 nt DR sequences flanking the spacer, iv) the secondary structure (determined using RNAfold online tool (Lorenz *et al.*, 2011)) of the DR sequences should remain intact and not interfere with the spacer (Creutzburg *et al.*, 2020) and v) off-targets at other non-intended genomic

locations should be avoided. The efficiency of the crRNA targeting can be affected by other parameters such as local chromatin structures, but the contribution of these factors is still poorly understood and they were not considered in the design process.

In case of *S. cerevisiae*, this study provides the IMX2713 strain expressing both endonucleases *Spy*Cas9 and *Er*Cas12a, which is a new platform strain for genetic modifications. The additional presence of Cas12a as editing tool makes the T-rich sequences more accessible for genetic editing, which could overcome the limitation of solely relying on the Cas9 editing strategy requiring a NGG PAM sequence. Since both endonucleases have proven to be very successful in targeting genomic DNA regions with their own PAM sequence specificity, together the targeting window of operation is almost the complete genome.

While plasmid-borne expression of *Er*cas12a was sufficient to edit *ADE2* with 31.5% efficiency, this constitutes a significant reduction from the 100% editing efficiency achieved with genome-integrated *Er*cas12a. Aside from *Er*Cas12a toxicity at high expression levels, another difference between plasmid-borne and genomic expression is the timing of the concurrent expression of *Er*Cas12a (transcription and translation), the editing event and the repair of the DSB with a linear repair DNA. While constitutive genomic expression ensures active *Er*Cas12a presence upon transformation of the crRNA-expressing plasmid and the dsDNA repair fragment, the expression of *Er*cas12a and of the gRNA from the same plasmid delays the time at which all three essential elements to perform the editing event are concomitantly present. To be optimal, the rate of the enzyme synthesis and gRNA expression must be fast and synchronized, while in the meantime the degradation of the linear repair DNA has to remain low. A too fast degradation of the linear repair DNA might explain this reduced efficiency. It is worth mentioning that similar construction using *Spy*Cas9 instead of *Er*Cas12a resulted in 100% efficiency (Generoso *et al.*, 2016), which might indicate a difference between Cas9 and Cas12a enzymes. Mitigation strategies to compensate this premature degradation could be beneficial such as the elongation of the repair (> 120bp used as default in this study), protection of the linear DNA by specific short sequences (Biswas *et al.*, 1995) or protein binding protection (Norouzi *et al.*, 2021). Despite a reduced editing efficiency, the transportable plasmid pUDP293 could be valuable for accessing multiple strains with different genetic background.

In conclusion, *Er*Cas12a was established as part of the *S. cerevisiae* genetic toolbox, able to edit several commonly used integration sites with (near) 100% efficiency in a single editing event as well as in multiplex editing. The single crRNAs or multi-spacer crRNA arrays could be ordered as two short annealing oligonucleotides and could easily and rapidly be cloned in purposed plasmids. The presented crRNA design criteria in combination with the developed genome editing strategy for *Er*Cas12a was shown to expand the genetic toolbox for editing of the yeast genome.

Data availability

Supplementary information can be accessed via the online publication.

Acknowledgements

We gratefully acknowledge Dr. Anna Wronska and Dr. Jasmijn Hassing for constructing strain IMX2600, Laura Sierra Herras for constructing the *Xdcrt* expressing plasmids pUD1248, pUD1249 and pUD1250 and Marcel van den Broek for assisting the genome analysis of the strains sequenced in the study. We are thankful to Prof. J.T. Pronk (Delft University of Technology), Dr. V.M. Boer and Mr T. Elink Schuurman (HEINEKEN Supply Chain B.V.) for their support during this project.

3.5. References

Babaei M, Sartori L, Karpukhin A, Abashkin D, Matrosova E & Borodina I (2021) Expansion of EasyClone-marker free toolkit for *Saccharomyces cerevisiae* genome with new integration sites. *FEMS Yeast Research* **21**.

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA & Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**: 1709-1712.

Biswas I, Maguin E, Ehrlich SD & Gruss A (1995) A 7-base-pair sequence protects DNA from exonucleolytic degradation in *Lactococcus lactis*. *Proceedings of the National Academy of Sciences* **92**: 2244-2248.

Botman D, de Groot DH, Schmidt P, Goedhart J & Teusink B (2019) *In vivo* characterisation of fluorescent proteins in budding yeast. *Scientific Reports* **9**: 2234.

Brouns SJJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJH, Snijders APL, Dickman MJ, Makarova KS, Koonin EV & van der Oost J (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**: 960-964.

Capecchi MR (1989) Altering the genome by homologous recombination. *Science* **244**: 1288-1292.

Cong L, Ran FA, Cox D, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**: 819-823.

Creutzburg SCA, Wu WY, Mohanraju P, Swartjes T, Alkan F, Gorodkin J, Staals RHJ & van der Oost J (2020) Good guide, bad guide: spacer sequence-dependent cleavage efficiency of Cas12a. *Nucleic Acids Research* **6**:3228-3243.

Danecek P, Bonfield JK, Liddle J, et al. (2021) Twelve years of SAMtools and BCFtools. *Gigascience* **10**.

DiCarlo JE, Norville JE, Mali P, Rios X, Aach J & Church GM (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research* **41**: 4336-4343.

Dorfman BZ (1969) The isolation of adenylosuccinate synthetase mutants in yeast by selection for constitutive behavior in pigmented strains. *Genetics* **61**: 377-389.

Edraki A, Mir A, Ibraheim R, et al. (2019) A compact, high-accuracy Cas9 with a dinucleotide PAM for *in vivo* genome editing. *Molecular Cell* **73**: 714-726 e714.

Entian KD & Kotter P (2007) Yeast genetic strain and plasmid collections. *Method Microbiology* **36**: 629-666.

Feng Z, Zhang B, Ding W, et al. (2013) Efficient genome editing in plants using a CRISPR/Cas system. *Cell Research* **23**: 1229-1232.

Flagfeldt D, Siewers V, Huang L & J N (2009) Characterization of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*. *Yeast* **26**: 545–551.

Gao Y & Zhao Y (2014) Self-processing of ribozyme-flanked RNAs into guide RNAs *in vitro* and *in vivo* for CRISPR-mediated genome editing. *Journal Integrative Plant Biology* **56**: 343-349.

Garneau JE, Dupuis MÈ, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH & Moineau S (2010) The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**: 67-71.

Generoso WC, Gottardi M, Oreb M & Boles E (2016) Simplified CRISPR-Cas genome editing for *Saccharomyces cerevisiae*. *Journal of Microbiol Methods* **127**: 203-205.

Gietz D, St Jean A, Woods RA & Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research* **20**: 1425.

Gill RT, Garst A & Warnecke LTE (2018) Nucleid acid-guided nucleases. Vol. US 9,982,279 B1 (Inscripta I, ed.) USA.

Gorter de Vries AR, de Groot PA, van den Broek M & Daran J-MG (2017) CRISPR-Cas9 mediated gene deletions in lager yeast *Saccharomyces pastorianus*. *Microbial Cell Factories* **16**: 222.

Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z & Liu DR (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* **556**: 57-63.

Ishino Y, Shinagawa H, Makino K, Amemura M & Nakata A (1987) Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology* **169**: 5429-5433.

Jarczynska ZD, Rendsvig JKH, Pagels N, Viana VR, Ndvig CS, Kirchner FH, Strucko T, Nielsen ML & Mortensen UH (2021) DIVERSIFY: A fungal multispecies gene expression platform. *ACS Synthetic Biology* **10**: 579-588.

Jiang W, Bikard D, Cox D, Zhang F & Marraffini LA (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology* **31**: 233-239.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA & Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816-821.

Juergens H, Varela JA, Gorter de Vries AR, Perli T, Gast VJM, Gyurchev NY, Rajkumar AS, Mans R, Pronk JT, Morrissey JP & Daran J-MG (2018) Genome editing in *Kluyveromyces* and *Ogataea* yeasts using a broad-host-range Cas9/gRNA co-expression plasmid. *FEMS Yeast Research* **18**.

Kalderon D, Roberts BL, Richardson WD & Smith AE (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39**: 499-509.

Kuijpers NGA, Solis-Escalante D, Bosman L, van den Broek M, Pronk JT, Daran J-M & Daran-Lapujade P (2013) A versatile, efficient strategy for assembly of multi-fragment expression vectors in *Saccharomyces cerevisiae* using 60 bp synthetic recombination sequences. *Microbial Cell Factories* **12**: 47-47.

Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeldnes H & Valen E (2019) CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research* **47**: W171-W174.

Ledford H (2022) Major CRISPR patent decision won't end tangled dispute. *Nature* **603**: 373-374.

Lee ME, DeLoache WC, Cervantes B & Dueber JE (2015) A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synthetic Biology* **4**: 975-986.

Li H (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv: *Genomics*.

Li Y, Pan S, Zhang Y, Ren M, Feng M, Peng N, Chen L, Liang YX & She Q (2015) Harnessing Type I and Type III CRISPR-Cas systems for genome editing. *Nucleic Acids Research* **44**.

Liachko I & Dunham MJ (2014) An autonomously replicating sequence for use in a wide range of budding yeasts. *FEMS Yeast Research* **14**: 364-367.

Lin Q, Zhu Z, Liu G, et al. (2021) Genome editing in plants with MAD7 nuclease. *Journal of Genetics and Genomics* **48**: 444-451.

Liu RM, Liang LL, Freed E, Chang H, Oh E, Liu ZY, Garst A, Eckert CA & Gill RT (2019) Synthetic chimeric nucleases function for efficient genome editing. *Nature Communications* **10**: 5524.

Liu Z, Schiel JA, Maksimova E, Strezoska Ž, Zhao G, Anderson EM, Wu Y, Warren J, Bartels A, Van Brabant Smith A, Lowe CE & Forbes KP (2020) *Er*Cas12a CRISPR-MAD7 for model generation in human cells, mice, and rats. *CRISPR Journal* **3**: 97-108.

Lõoke M, Kristjuhan K & Kristjuhan A (2011) Extraction of genomic DNA from yeasts for PCR-based applications. *BioTechniques* **50**: 325-328.

Lorenz R, Bernhart SH, Honer Zu Siederdissen C, Tafer H, Flamm C, Stadler PF & Hofacker IL (2011) ViennaRNA Package 2.0. *Algorithms for Molecular Biology* **6**: 26.

Makarova KS, Wolf YI, Iranzo J, et al. (2020) Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. *Nature Research* **18**: 67-83.

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE & Church GM (2013) RNA-guided human genome engineering via Cas9. *Science* **339**: 823-826.

Mans R, van Rossum HM, Wijsman M, Backx A, Kuijpers NG, van den Broek M, Daran-Lapujade P, Pronk JT, van Maris AJ & Daran JM (2015) CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Research* **15**.

Marrafini L & Sontheimer E (2009) CRISPR interference limits horizontal gene transfer in *Staphylococci* by targeting DNA. *Science* **322**: 1843-1845.

Mikkelsen MD, Buron LD, Salomonsen B, Olsen CE, Hansen BG, Mortensen UH & Halkier BA (2012) Microbial production of indolylglucosinolate through engineering of a multi-gene pathway in a versatile yeast expression platform. *Metabolic Engineering* **14**: 104-111.

Mizuno S, Dinh TTH, Kato K, Mizuno-Iijima S, Tanimoto Y, Daitoku Y, Hoshino Y, Ikawa M, Takahashi S, Sugiyama F & Yagami KI (2014) Simple generation of albino C57BL/6J mice with G291T mutation in the tyrosinase gene by the CRISPR/Cas9 system. *Mammalian Genome* **25**: 327-334.

Mojica FJM, Juez G & Rodriguez-Valera F (1993) Transcription at different salinities of *Haloferax mediterranei* sequences adjacent to partially modified PstI sites. *Molecular Microbiology* **9**: 613-621.

Mojica FJM, Díez-Villaseñor C, García-Martínez J & Soria E (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution* **60**: 174-182.

Norouzi M, Panfilov S & Pardee K (2021) High-efficiency protection of linear DNA in cell-free extracts from *Escherichia coli* and *Vibrio natriegens*. *ACS Synthetic Biology* **10**: 1615-1624.

Postma ED, Dashko S, van Breemen L, Taylor Parkins SK, van den Broek M, Daran J-M & Daran-Lapujade P (2021) A supernumerary designer chromosome for modular *in vivo* pathway assembly in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **49**: 1769-1783.

Price MA, Cruz R, Bryson J, Escalettes F & Rosser SJ (2020) Expanding and understanding the CRISPR toolbox for *Bacillus subtilis* with MAD7 and dMAD7. *Biotechnology and Bioengineering* **117**: 1805-1816.

Randazzo P, Bennis NX, Daran J-M & Daran-Lapujade P (2021) gEL DNA: A cloning- and polymerase chain reaction–free method for CRISPR-based multiplexed genome editing. *The CRISPR Journal* **4**: 896-913.

Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G & Mesirov JP (2011) Integrative genomics viewer. *Nature Biotechnology* **29**: 24-26.

Rojek J, Basavaraju Y, Nallapareddy S, Baumgartner R, Schoffelen S & Pedersen L (2021) Mad7: An IP friendly CRISPR enzyme. *Authorea*.

Rojek J, Basavaraju Y, Nallapareddy S, Baumgartner RF, Schoffelen S & Pedersen LE (2021) Mad7: An IP Friendly CRISPR Enzyme. *Research Square*.

Sapranauskas R, Gasiunas G, Fremaux C, Barrangou

R, Horvath P & Siksnys V (2011) The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Research* **39**: 9275-9282.

Shaffer C (2022) Broad defeats Berkeley CRISPR patent. *Nature Biotechnology* **40**: 445.

Swarts DC & Jinek M (2018) Cas9 versus Cas12a/Cpf1: Structure–function comparisons and implications for genome editing. *WIREs RNA* **9**: e1481.

Swiat MA, Dashko S, den Ridder M, Wijsman M, van der Oost J, Daran JM & Daran-Lapujade P (2017) *Fn*Cpf1: a novel and efficient genome editing tool for *Saccharomyces cerevisiae*. *Nucleic Acids Research* **45**: 12585-12598.

van den Broek M, Ortiz-Merino RA, Bennis NX, Wronska AK, Hassing EJ, Daran-Lapujade P & Daran JM (2024) Draft genome sequence of the *Saccharomyces cerevisiae Spy*Cas9 expressing strain IMX2600, a laboratory and platform strain from the CEN.PK lineage for cell-factory research. *Microbiological Resource Announcement* **13**: e0055023.

Vanegas KG, Rendsvig JKH, Jarczynska ZD, Cortes MVdCB, van Esch AP, Morera-Gómez M, Contesini FJ & Mortensen UH (2023) A Mad7 system for genetic engineering of filamentous fungi. *Journal of Fungi* **9**: 16.

Verduyn C, Postma E, Scheffers WA & Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**: 501-517.

Verwaal R, Buiting-Wiessenhaan N, Dalhuijsen S & Roubos JA (2018) CRISPR/Cpf1 enables fast and simple genome editing of *Saccharomyces cerevisiae*. *Yeast* **35**: 201-211.

Verwaal R, Wang J, Meijnen JP, Visser H, Sandmann G, van den Berg JA & van Ooyen AJ (2007) High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. *Applied Environmental Microbiology* **73**: 4342-4350.

Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, Cuomo CA, Zeng Q, Wortman J, Young SK & Earl AM (2014) Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLOS ONE* **9**: e112963.

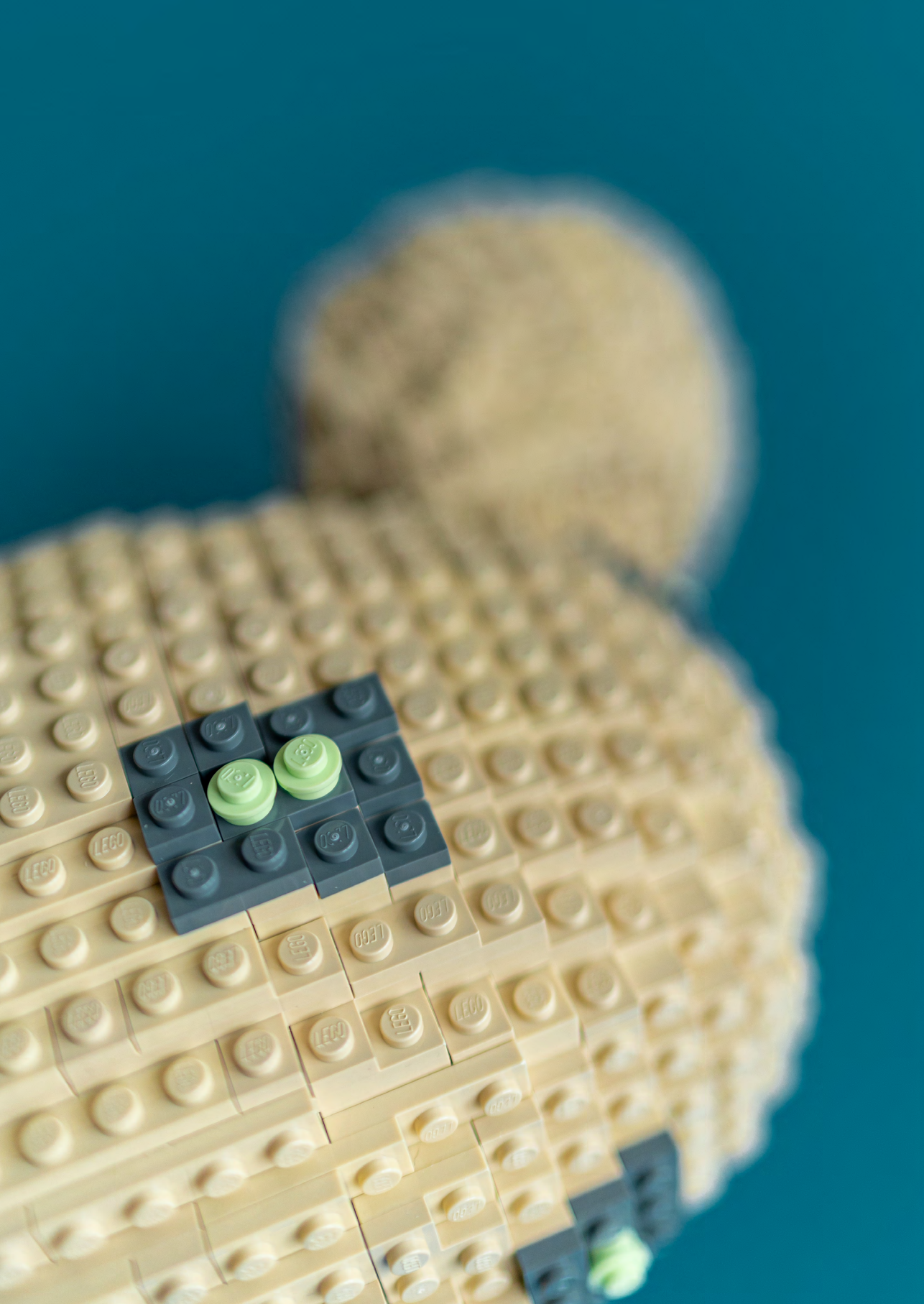
Wierson WA, Simone BW, WareJoncas Z, Mann C, Welker JM, Kar B, Emch MJ, Friedberg I, Gendron WAC, Barry MA, Clark KJ, Dobbs DL, McGrail MA, Ekker SC & Essner JJ (2019) Expanding the CRISPR toolbox with *Er*Cas12a in zebrafish and human cells. *The CRISPR Journal* **2**: 417-433.

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV & Zhang F (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**: 759-771.

Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, Wu WY, Scott DA, Severinov K, van der Oost J & Zhang, F. (2017) Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nature Biotechnology* **35**: 178-178.

Zhang XH, Tee LY, Wang XG, Huang QS & Yang SH (2015) Off-target effects in CRISPR/Cas9-mediated genome engineering. *Molecular Therapy Nucleic Acids* **4**: e264.

Zhang Y, Ren Q, Tang X, *et al.* (2021) Expanding the scope of plant genome engineering with Cas12a orthologs and highly multiplexable editing systems. *Nature Communications* **12**.



Chapter 4

Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts

Nicole X. Bennis, Matic Kostanjšek, Marcel van den Broek & Jean-Marc G. Daran

This chapter has been published in New Biotechnology (2022)

Abstract

Saccharomyces pastorianus is not a classical taxon, it is an interspecific hybrid resulting from the cross of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus*. Exhibiting heterosis for phenotypic traits, such as wort α-oligosacharides consumption and fermentation at low temperature, it has been domesticated to become the main workhorse of the brewing industry. Although CRISPR-Cas9 has been shown to be functional in *S. pastorianus*, repair of CRISPR induced double strand break is unpredictable and preferentially uses the homoeologous chromosome as template preventing targeted introduction of the desired repair construct. Here, we demonstrate that lager hybrids can be edited with near 100% efficiency at carefully selected landing sites on the chimeric *Se*Sc*CHRIII*. The landing sites were systematically selected and evaluated for i) absence of loss of heterozygosity upon CRISPR-editing, ii) efficiency of the gRNA, and iii) absence of effect on strain physiology. Successful examples of highly efficient single and double gene integration illustrated that genome editing can be applied in interspecies hybrids, paving the way to a new impulse to lager yeast strain development.

4.1. Introduction

With an annual production of almost 2 billion hectolitres, lager beer is the worlds most consumed alcoholic beverage (https://www.statista.com/). The workhorse for lager beer production is the yeast *Saccharomyces pastorianus*, a bottom fermenting hybrid yeast that has emerged from a natural hybridization event, probably occurring during the Middle Ages, between the mesophilic ale yeast *Saccharomyces cerevisiae* and the cryotolerant yeast *Saccharomyces eubayanus* (Nakao *et al.*, 2009, Libkind *et al.*, 2011, Bergin *et al.*, 2022). Hitherto the almost entire research aiming to improve the brewing performance of lager strains is based on classical strain improvement methods, such as mutagenesis and selection, crossing and breeding, and laboratory evolution (Blieck *et al.*, 2007, Ekberg *et al.*, 2013, Steensels *et al.*, 2014, Krogerus *et al.*, 2015, Brickwedde *et al.*, 2017, Gorter de Vries *et al.*, 2019, Gibson *et al.*, 2020, Iattici *et al.*, 2020). Brewing strains resulting from these techniques are regarded as non-genetically modified organisms. Although these techniques proved their efficiency, they are laborious, time consuming and unpredictable, therefore limiting the pace of strain development. Conversely, molecular techniques enabling introduction of targeted genetic modifications would offer a faster alternative to create specific mutants that might exhibit better brewing characteristics. The deployment of genetic engineering strain improvement strategies in brewing industry is, however, impeded by three factors. The first is the producers' concerns about consumer acceptance of beers brewed with genetically modified yeasts (Gorter de Vries *et al.*, 2019). While it is difficult to predict whether these reluctances will last, in North America, yeasts-producing companies are already marketing modified ale and even lager strains (https://berkeleyyeast.com/; https://www.lallemandbrewing.com/) demonstrating that mentalities are changing. The second point concerns the limited genetic accessibility of these hybrid yeasts, a trait likely related to the complex structure of their genome. Thirdly, the breweries have to deal with the regulatory implications for production with genetically modified organisms (GMOs), depending on the location in the world.

The hybrid nature of the *S. pastorianus* genome was confirmed for the first time with the genome sequence of the *S. pastorianus* Weihenstephan 34/70 strain (Nakao *et al.*, 2009). This initial work was followed by multiple other *S. pastorianus* strain sequencing projects (Walther *et al.*, 2014, van den Broek *et al.*, 2015, Salazar *et al.*, 2019, Turgeon *et al.*, 2021) that confirmed that lager yeast harbored an allo-aneuploid genome. Although strain to strain variations exist, lager yeast genome comprises both parental chromosome sets which all have different copy numbers. In addition to the presence of multiple copies, each chromosome has a homoeologous chromosome, which is derived from the other parental species (Glover *et al.*, 2016). For example, the *S. cerevisiae* CHRI is homoeolog to the *S. eubayanus* CHRI which shares both high sequence similarity and gene syntheny (Brouwers *et al.*, 2019, Salazar *et al.*, 2019). This co–occurrence has likely contributed to translocation and loss of heterozygosity

(LOH) events between chromosomes of the two subgenomes (*ScSe* and *SeSc*) in *S. pastorianus* that are considered as hallmarks of domestication (van den Broek *et al.*, 2015, Gorter de Vries *et al.*, 2020).

While this dual genome composition has promoted the emergence of essential phenotypic traits of lager brewing yeasts, such as ability to flocculate, to rapidly ferment sugars (including α-oligoglucosides) into ethanol, to produce a variety of flavour and aroma compounds and to ferment at low temperatures among the most relevant, presence of homoeologous chromosome pairs have been impairing genetic amenability (Gorter de Vries *et al.*, 2018). This effect is even stronger when genome editing tools such as CRISPR-Cas9 are used. CRISPR-Cas endonucleases can target specific DNA sequences based on the user-defined composition of the single guide RNA (gRNA) sequence and presence of the protospacer adjacent motif (PAM) (Brouns *et al.*, 2008, Al-Attar *et al.*, 2011, Jinek *et al.*, 2012, Charpentier & Doudna, 2013). The CRISPR-Cas induced double strand breaks (DSB) are extremely recombinogenic and are resolved by the yeast homology directed repair (HDR) machinery that uses native or foreign DNA containing sequence homology as repair template, thereby producing the desired genetic edit (DiCarlo *et al.*, 2013).

Although the CRISPR-Cas9 methodology has been successfully applied in *S. pastorianus* (Gorter de Vries *et al.*, 2017), it appeared in many situations that the editing outcome is unpredictable and characterization of the resulting edited locus has been proven troublesome (Gorter de Vries *et al.*, 2018). Whereas in homozygous diploid yeasts the gRNA programmed Cas9 introduces DSB on both copies, in hybrid yeasts, gRNA are not necessarily designed to cut at all parental alleles. In this latter situation, the uncut homoeologous locus competes with the provided repair DNA fragments, facilitating chromosome recombination and reduced editing efficiency, resulting in loss of heterozygosity and even more undesired genetic changes (Gorter de Vries *et al.*, 2018).

In this study, we aim to improve the genetic accessibility of the allo-aneuploid yeast *S. pastorianus* by identifying unique landing sites devoid of homoeologous regions using a systematic approach. We evaluate whether our approach eliminates loss of heterozygosity when performing precise genome editing with CRISPR-Cas9. Identifying and targeting unique sequences offers opportunities for increased genetic engineering efficiencies in allo-aneuploid yeast. To demonstrate this, integration efficiency of single or double genes will be tested.

4.2. Materials and Methods

Strains and cultivation conditions

All yeast strains used in this study are listed in Table 4.1. Yeast were grown on complex medium that contained 10.0 g L^{-1} bacto yeast extract, 20.0 g L^{-1} bacto peptone and

Table 4.1. Strains used in this study

Species*	Strain	Relevant genotype	Origin
Sc	CEN.PK113-7D	*MATa MAL2–8c SUC2*	(Entian & Kötter, 2007)
Sp	CBS 1483	Group II brewer's yeast, Heineken bottom yeast, isolated July 1927	(Dunn & Sherlock, 2008)
Sp	WS 34/70	Group II brewer's yeast, flocculent yeast	(Nakao et al., 2009)
Sp	IMK1062	CBS 1483 Δ*ScEEB1*::ymNeongreen (LOH), colony 1	This study
Sp	IMK1063	CBS 1483 Δ*ScEEB1*::ymNeongreen (LOH), colony 3	This study
Sp	IMK1064	CBS 1483 Δ*ScEEB1*::ymNeongreen (LOH), colony 4	This study
Sp	IMK1065	CBS 1483 Δ*ScEEB1*::ymNeongreen (LOH), colony 6	This study
Sp	IMI504	CBS 1483 Δ*Se*YCL049C::ymNeongreen	This study
Sp	IMI505	CBS 1483 Δ*Se*YCL036W::ymNeongreen	This study
Sp	IMI506	CBS 1483 Δ*Se*YCL012C::ymNeongreen	This study
Sp	IMI507	CBS 1483 ΔScYCR051W::ymNeongreen	This study
Sp	IMI508	CBS 1483 ΔScYCR087C-A::ymNeongreen	This study
Sp	IMI509	CBS 1483 Δ*Se*Site1::ymNeongreen	This study
Sp	IMI510	CBS 1483 Δ*Se*Site2::ymNeongreen	This study
Sp	IMI511	CBS 1483 Δ*Se*Site3::ymNeongreen	This study
Sp	IMI512	CBS 1483 Δ*Se*Site4::ymNeongreen	This study
Sp	IMI483	CBS 1483 ΔScYCR087C-A::*pTDH3-BbaldC-tENO1*	This study
Sp	IMI485	CBS 1483 ΔScYCR087C-A::*pTDH3-LlaldC-tENO1*	This study

***** *Sc* is referring to *S. cerevisiae* and *Sp* is referring to *S. pastorianus*.

20 g L^{-1} glucose (YPD). For selection of transformants or when selective pressure was required, YPD medium was supplemented with 200 mg·L-1 of hygromycin (YPD hygR). Solid YPD media was obtained by addition of 20 g L^{-1} bacto agar. *S. cerevisiae* and *S. pastorianus* strains were cultivated at 30 °C and 20 °C, respectively while shaking at 200 rpm in an Innova 43/43R incubation shaker (Brunswick, Nijmegen, the Netherlands). On solid media, *S. cerevisiae* strains were cultivated on agar plates at 30 °C in a stationary incubator (Bronson incubator services B.V., Zaltbommel, the Netherlands) whereas *S. pastorianus* strains were incubated at 20 °C in a temperature controlled room. Fermentations were performed with full malt wort at either 17 °P or 5.7 °P (Heineken, Zoeterwoude, The Netherlands) supplemented with 1 mL L^{-1} pluronic acid.

Table 4.2. Plasmids used in this study.

Plasmid	Genotype	Reference
pUDP002	*ori ampR* panARS^{OPT} *AgTEF1p-hph-AgTEF1t ScTDH3p-*$^{BsaI\_BsaI}$*-ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	(Juergens *et al.*, 2018)
pUD1205	*ori kanR* ^{BsaI}HH-gRNA *Se*YCL049C-HDV^{BsaI}	GeneArt
pUD1206	*ori kanR* ^{BsaI}HH-gRNA *Se*YCL036W-HDV^{BsaI}	GeneArt
pUD1207	*ori kanR* ^{BsaI}HH-gRNA *Se*YCL012C-HDV^{BsaI}	GeneArt
pUD1208	*ori kanR* ^{BsaI}HH-gRNA *Sc*YCR051W-HDV^{BsaI}	GeneArt
pUD1209	*ori kanR* ^{BsaI}HH-gRNA *Sc*YCR087C-A-HDV^{BsaI}	GeneArt
pUD1243	*ori kanR* ^{BsaI}HH-gRNA *Se*Site1-HDV^{BsaI}	GeneArt
pUD1244	*ori kanR* ^{BsaI}HH-gRNA *Se*Site2-HDV^{BsaI}	GeneArt
pUD1212	*ori kanR* ^{BsaI}HH-gRNA *Se*Site3-HDV^{BsaI}	GeneArt
pUD1213	*ori kanR* ^{BsaI}HH-gRNA *Se*Site4-HDV^{BsaI}	GeneArt
pUDP168	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *ScEEB1*-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP172	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *ScEAT1*-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP269	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Se*YCL049C-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP270	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Se*YCL036W-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP271	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Se*YCL012C-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP272	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Sc*YCR051W-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP273	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Sc*YCR087C-A-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP283	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Se*Site1-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP284	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Se*Site2-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP276	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Se*Site3-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pUDP277	*ori ampR* panARS$^{(OPT)}$ *AgTEF1p-hph-AgTEF1t ScTDH3p*-HH-gRNA *Se*Site4-HDV-*ScCYC1t AaTEF1p-Spcas9*$^{D147Y\ P411T}$*-ScPHO5t*	This study
pYTK009	*ori camR pTDH3*	(Lee *et al.*, 2015)
pYTK051	*ori camR tENO1*	(Lee *et al.*, 2015)
pYTK053	*ori camR tADH1*	(Lee *et al.*, 2015)

Table 4.2. (continued)

Plasmid	Genotype	Reference
pGGKp302	*ori camR* ymScarletI	(Botman *et al.*, 2019)
pGGKp304	*ori camR* ymNeongreen	(Botman *et al.*, 2019)
pGGKd015	*ori ampR* ConLS-GFPdo-ConR1	(Hassing *et al.*, 2019)
pGGKd034	*ori ampR* 2µm *hygR* ConLS-GFPdo-ConR1	This study
pUDE1111	*ori ampR* 2µm *hygR* ConLS-*pTDH3*-ymNeongreen-*tADH1*-ConR1	This study
pUDE1112	*ori ampR* 2µm *hygR* ConLS-*pTDH3*-ymScarletI-*tADH1*-ConR1	This study
pUD374	*ori kanR BbaldC*	This study
pUD376	*ori kanR LlaldC*	This study
pUD1218	*ori ampR pTDH3-BbaldC-tENO1*	This study
pUD1220	*ori ampR pTDH3-LlaldC-tENO1*	This study

Escherichia coli XL1-Blue (New England Biolabs, Ipswich, MA, USA) cultures were grown in 15 mL Greiner tubes containing 5 mL lysogenic broth (LB) medium supplemented with 10 mg L^{-1} ampicillin (LB Amp), 50 mg L^{-1} kanamycin (LB Kan) or 10 mg L^{-1} chloramphenicol (LB Cam) for selection and cultivated at 37 °C while shaking at 200 rpm in an Innova 4000 Incubator Shaker (Eppendorf AG, Hamburg, Germany). Solid medium was prepared by addition of 20 g L^{-1} bacto agar to the medium.

Frozen stock cultures of *S. cerevisiae*, *S. pastorianus* and *E. coli* strains were prepared by addition of 30% (v/v) glycerol and stocks were stored as 1 mL aliquots at -80 °C.

Molecular biology techniques

PCR amplifications for cloning purposes were performed using Phusion High Fidelity Polymerase (Thermo Fisher Scientific, Landsmeer, The Netherlands) according to the manufacturer's instructions. Diagnostic PCRs were performed with DreamTaq PCR Mastermix (Thermo Fisher Scientific) according to the manufacturer's instructions. All plasmids and primers (Sigma Aldrich, Zwijndrecht, The Netherlands) constructed or used in this study are listed in Table 4.2 and Table 4.3, respectively. DNA was visualized by electrophoresis on 1% agarose gels with the GeneRuler™ Ladder Mix (Thermo Scientific) for size verifications. PCR products were purified from gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Alternatively, PCR mixtures were digested with FastDigest DpnI enzyme (Thermo Fisher Scientific) to remove circular templates. Digested PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) following

Table 4.3. Primers used in this study.

#	Sequence (5' -> 3')	Purpose
gRNA plasmid construction		
4068	GCCTACGGTTCCCGAAGTATGC	Diagnostic primer gRNA cloning into pUDP002
18516	TGTCTCTGACTGTATCTGGA	Diagnostic primer gRNA YCL049C
18521	GCGACTCCTCAATGATCAAA	Diagnostic primer gRNA YCL036W
18526	CGTCAATATAACTACATTTTGGGA	Diagnostic primer gRNA YCL012C
18531	ATACCCCGTTGCACCATG	Diagnostic primer gRNA YCR051W
18536	CACGCCATCTTCAAACGTCT	Diagnostic primer gRNA YCR087C-A
19067	CGTATAGCACTCCTGCCGAA	Diagnostic primer gRNA Site 1
19068	ACTACCCCTAGCACTGCTCA	Diagnostic primer gRNA Site 2
18551	GTCGAGAAGATTTCCTGAAGATA	Diagnostic primer gRNA Site 3
18556	CGTCATAATGAACAATCTCCAGTG	Diagnostic primer gRNA Site 4
1153	GACCCACGCATGTATCTATCTC	Diagnostic primer gRNA *ScEEB1* & *ScEAT1*
580	*GAATGTAAGCGTGACATAAC*	Diagnostic primer gRNA *ScEEB1* & *ScEAT1*
Construction expression cassettes (ymNeongreen, ymScarletI, *BbALC* and *LlALDC*)		
10320	CATGCGCGGATGACACGAAC	Diagnostic primer YTK
10325	AGTCATCCGAGCGTGTATTG	Diagnostic primer YTK
18759	AAAGCATCGTCTCATCGGTCTCATATGACTACTGCTACT-GTTCCAGC	Amplification *BbALDC*
18760	AAAATGCCGTCTCAGGTCTCAGGATAGT-TACTTTCTTTCAGATTCAGCTTGGTG	
18763	AAAGCATCGTCTCATCGGTCTCATATGTCTAGATTGTAC-CAACACGGTAC	Amplification *LlALDC*
18764	AAAATGCCGTCTCAGGTCTCAGGATAGTTATTGTTGAC-CACCTTCAGAAGC	

Table 4.3. (continued)

#	Sequence (5' -> 3')	Purpose
Construction integration fragments		
18512	TTCTATAGATGTACGTAAAGTTCT-GCTCTTCTTTTATAGATAAGAGGATTGAGACTCGCCACT-GGCCGATAATTGCAGACG	Integration in *Se*YCL049C
18513	AGCTCAAGAAAAACCAGGCTATTCGCAGAACAGGA-TAACCAGCTCTGTAACCACATCAATCATGATGAGCCGT-GATGACCC	
18517	AGATGCGGCAGCCAGTGAGGGCGTTGGGCATGATC-GAAAGCCAAGACCCACCAATTCGAGACTGGCCGATA-ATTGCAGACG	Integration in *Se*YCL036W
18518	ATATACATATACGTACGTATGTATCTA-CAGAAAGAAAAAAAAAAAAGATCAATGAATATATCAT-GATGAGCCGTGATGACCC	
18522	CAAATTTGAAAGGAACTAAAAAGACAGGAGGAACCTC-CCCTTTGTATGAGCTCAAAATAAACTGGCCGATAATTG-CAGACG	Integration in *Se*YCL012C
18523	CCTCCTCATTCAAGGAAGAGAAATGAAAATACTTCTTG-CAAGAAGGTTGCAAATACTATACATGATGAGCCGTGAT-GACCC	
18527	GAGAACAAGAAGAGTTTGCAGGTGACAAAAATCGAT-GATTATAGGTGTTGTGACGACAAAACTGGCCGATA-ATTGCAGACG	Integration in *Sc*YCR051W
18528	TTCATGTAGCGTCGCGTTCAATTTCTTTTAGCAAGC-TATAAGAGCCTTTGTGCTGGGTCGCATGATGAGCCGT-GATGACCC	
18532	AAAAGATGAAACCGAGTAAGCTGCTACATAATGTC-TATATATCTACACATAAAATTCCGAACTGGCCGATA-ATTGCAGACG	Integration in *Sc*YCR087C-A
18533	TAAGAGTATTCTGTATACAACAGCAAACGGTCTCAGT-CAAGAAATATTTGTTATTACAGGCATGATGAGCCGT-GATGACCC	
18537	CCTCCGCTATCTAGATGGATACAGAAAGCCGTTTTTTG-GTGATTATGTTCACTGTCAAGTACTGGCCGATAATTG-CAGACG	Integration in *Se*Site1
18538	CTTAAATTGTGGAAGACTCCATCCAAGAGAAGGATCAG-GTATCTGAATTATTCTAAAAGCCATGATGAGCCGTGAT-GACCC	
18542	ACTCGATGACTCACAGGACAGGAGCGCTTTAAA-CAAGACGGAATACATAAGCGCATGTGCACTGGCCGA-TAATTGCAGACG	Integration in *Se*Site2
18543	AAATTACTTCTTGTCATTATAAAAAGACTAGTACGCTTC-TATACGTATATTTATTCTCTACATGATGAGCCGTGAT-GACCC	

Table 4.3. (continued)

#	Sequence (5' -> 3')	Purpose
18547	AAAGCACACGCTGATTGGTTATATGACACCCAATGGTA-AAAGTAAGGTTCAGAACATTAAACTGGCCGATAATTG-CAGACG	Integration in *Se*Site3
18548	ATATCTCTTAGGCCAGAATATGTTATTTAACCGTTA-AAAGCACTTAGAAGATGACAGGGCCATGATGAGCCGT-GATGACCC	
18552	CCAATCAGCGTGTGTTTTATATACCTCTCTTATATA-ATTTAAGAAAGAACTGCTTATTCTACTGGCCGATAATTG-CAGACG	Integration in *Se*Site4
18553	TGTTGGTTTTATAAGCGCATTGATTGTTTGTTTGA-GAGTCTTAAAGTCAAATAACACCTGCATGATGAGCCGT-GATGACCC	
18981	GGTAGAGAAAGTGGATCGGCGGATTGGAAAGCACAG-CGTGGGGAGGATGGTAAATAGAGAACTGGCCGATA-ATTGCAGACG	Integration in *ScEEB1*
18982	TTTTCGGTATTTTTGAAGATTAGCAAAAAGATCAAGA-TATCAAGTATTTTCATATTTGTCCATGATGAGCCGTGAT-GACCC	
18985	TGCTGTGTGCGTTGATTTGGGCCTGACAC-GAGAAGAAAGTGCGTTACGTACATCAAGATTACTGGC-CGATAATTGCAGACG	Integration in *ScEAT1*
18986	CCAAGGTCGAGACGTATACAAACTGCAAAATAAAAG-GAACCGTGGGAAGAGGGCTTACAACATGATGAGCCGT-GATGACCC	
18768	CGGTCAGATGGGATACAATCTAGATAAGTTGCGCTG-TAGCAGCAAGCTGAATAGCGATGCCATGATGAGCCGT-GATGACCC	*In vivo* assembly SHR CB flank incorporation
13047	GCATCGCTATTCAGCTTGCTGCTACAGCGCAACT-TATCTAGATTGTATCCCATCTGACCGGCCGATAATTG-CAGACGAAC	
Diagnostic primers for integration verification		
18514	TCAGTTAGAGTGACAGTTGC	Integration in *Se*YCL049C
18515	GTTGTGCGTTTTACGTGC	
18519	TCCGTCAGTATTCGAGGC	Integration in *Se*YCL036W
18520	TCTTGGAACCTATCCTGGC	
18990	GGCTCGAATTTTATTGAGTGG	Integration in *Se*YCL012C
18991	GGTATCTAAAATGCGTTCAAGG	
18529	CCATTCATTGTTTAAGTTTCGGG	Integration in *Sc*YCR051W
18530	GCTTTTCTTTCACTCTACAACG	
18534	GAATACCTCTTCGAAACGTTGAG	Integration in *Sc*YCR087C-A
18535	ATGAGTGGACTGGCAGC	
18539	GGACATGAAAGAGCCCAG	Integration in *Se*Site1
18540	CTCAAACGCTACAAAGGAAGC	

Table 4.3. (continued)

#	Sequence (5' -> 3')	Purpose
18544	CGTTGGAAAGAGTTGTTACTTTCTG	Integration in *Se*Site2
18545	GCTTTACACTGATATCAAATAGCC	
18549	GAGGTCAAAATGTTGATAATTAGGAG	Integration in *Se*Site3
18550	GCATTTGTAATATTTCCAAGCTGC	
18992	GGAATATGTGACTTCGGGC	Integration in *Se*Site4
18993	ACCTAATCAACGTGAAGGC	
15944	AGTGCCGCTTCGAAATCATC	Integration in *ScEEB1*
15945	TTCTGCATCTGGTTGCCTAC	
15950	CTGGGTTGGAACGAAGTTTG	Integration in *ScEAT1*
15951	ACCGTCATGAGTGTAGTCAG	

the manufacturer's instructions using milliQ as eluent solvent. Golden Gate cloning was performed using 25 fmol of each fragment with BsmBI or BsaI restriction enzymes (New England Biolabs) according to (Engler *et al.*, 2008, Lee *et al.*, 2015). Gibson Assembly was performed with 200 fmol of each fragment using NEBuilder® HiFi DNA Assembly Master Mix (New England BioLabs) and one hour incubation at 50 °C (Gibson *et al.*, 2009). Constructed plasmids were transformed to *E. coli* XL1-blue (New England Biolabs) chemically competent cells. Plasmids were isolated from the *E. coli* cells with the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the supplier's manual with milliQ as eluent solvent.

CRISPR-Cas9 plasmid construction

The gRNA sequences for the introduction in Cas9 platform plasmid pUDP002 (Juergens *et al.*, 2018) were designed in such a way that they yield compatible sticky ends for ligation upon restriction with BsaI, following the Golden Gate cloning strategy for gRNA cloning described by Gorter de Vries *et al.*, 2017. The plasmids pUD1205-pUD1209, pUD1243, pUD1244, pUD1212 and pUD1213 (GeneArt, Thermo Fisher) contain the gRNA sequences targeting *Se*YCL049C, *Se*YCL036W, *Se*YCL012C, *Sc*YCR051W, *Sc*YCR087C-A, *Se*Site1, *Se*Site2, *Se*Site3 and *Se*Site4, respectively, and are flanked by the HammerHead (HH) and Hepatitis Delta Virus (HDV) ribozymes. Correct Golden Gate cloning of these gRNAs into the platform plasmid pUDP002 resulted in pUDP269-pUDP273, pUDP283, pUDP284, pUDP276 and pUDP277, which were verified by diagnostic PCR with primer 4068 binding to SHR I and primers 18516, 18521, 18526, 18531, 18536, 18541, 19067, 19068, 18551 and 18556 binding to the respective gRNA spacers.

Cloning of the gRNAs targeting *ScEEB1* and *ScEAT1* into pUDP002, resulting in pUDP168 and pUDP172 was confirmed in a diagnostic PCR with primers 1153 & 580.

The plasmid pUDE1111 and pUDE1112 that express the ymNeongreen gene and the

ymScarletI gene, respectively, were constructed using Golden Gate cloning with plasmids pYTK009, pYTK053 (Lee *et al.*, 2015), pGGKp304 (ymNeongreen (Botman *et al.*, 2019)) or pGGKp302 (ymScarletI (Botman *et al.*, 2019)) and pGGKd034 (Table 4.2). Correct construction of pUDE1111 and pUDE1112 was verified by diagnostic PCR with primers 10320 & 10325.

Construction of acetolactate decarboxylase gene expressing plasmids

aldC genes from *Brevibacillus brevis* (CP087980.1), excluding the secretion signal peptide, and *Leuconostoc lactis* (CP042420.1) were codon optimized for yeast, and ordered as synthetic genes (GeneArt, Thermo Fisher), resulting in plasmids pUD374 and pUD376. Genes were amplified from these plasmids using primer pairs 18759 & 18760 and 18763 & 18764, respectively, incorporating golden gate compatible flanks. Golden gate assembly with pGGKd015, pYTK009, pYTK051 and the PCR-amplified *aldC* gene fragment resulted in plasmids pUD1218 and pUD1220, respectively, for *BbaldC* and *LlaldC*, which were verified by diagnostic PCR using the primer pair 10320 & 10325.

CRISPR-Cas9 genome editing in *S. pastorianus* and *S. cerevisiae* strains

CRISPR-Cas9 genome editing in *S. pastorianus* strains was performed by transforming CBS 1483 or WS 34/70 with 500 ng gRNA plasmid and 1000 ng repair fragment (Table 4.4). As negative control, transformations from which the repair fragment was omitted were performed. Yeast transformation was performed by electroporation using 50 µL of competent cells in 0.2 cm chilled Gene Pulser® Cuvettes (Bio-Rad) and pulsed with for 5 ms with 1.5 kV by the MicroPulser (Serial No. 411BR5600, Bio-Rad). The transformed cells were incubated in 0.5 mL YPD during 2 h, followed by re-suspending in 100 µL sterile demi-water and plating on selective medium. CRISPR-Cas9 genome editing in *S. cerevisiae* strain CEN.PK113-7D was performed by transforming 500 ng gRNA plasmid and 1000 ng repair fragment according to the LiAc/ss-DNA/PEG chemical transformation method (Gietz & Woods, 2002).

The ymNeongreen integrations were performed by co-transforming the gRNA plasmid and the corresponding repair fragment consisting of the ymNeongreen expression cassette flanked by 60 bp homologous sequences for recombination. The ymNeongreen repair fragments were obtained by PCR amplification using pUDE1111 as template and primer pairs 18512 & 18513 for *Se*YCL049C, 18517 & 18518 for *Se*YCL036W, 18522 & 18523 for *Se*YCL012C, 18527 & 18528 for *Sc*YCR051W, 18532 & 18533 for *Sc*YCR087C-A, 18537 & 18538 for *Se*Site1, 18542 & 18543 for *Se*Site2, 18547 & 18548 for *Se*Site3 and 18552 & 18553 for *Se*Site4. For replacement of the *ScEEB1* and *ScEAT1* in *S. pastorianus* and *S. cerevisiae* strains, the ymNeongreen repair fragment was obtained via PCR amplification with primer pairs 18981 & 18982 and 18985 & 18986, respectively, with pUDE1111 as template. The *aldC* gene expression cassettes for integration in *Sc*YCR087C-A were obtained by PCR amplification with primers 18532 & 18533 using

pUD1218 or pUD1220, respectively, as template.

In vivo assembled integration of ymNeongreen and ymScarletI was performed with two fragments to repair the CRISPR-induced DSB. The ymNeongreen fragment was amplified from pUDE1111 with primers 18512 & 18768 incorporating the *Se*YCL049C flank and the SHR CB flank, while the ymScarletI fragment was amplified from pUDE1112 with primers 13047 & 18513 incorporating the SHR CB and *Se*YCL049C flanks.

Yeast genomic DNA for diagnostic purposes was isolated using the lithium acetate (LiOAc)-SDS method (Lõoke *et al.*, 2011). Gene integrations in *Se*YLC049C were confirmed with primers 18514 & 18515, *Se*YCL036W with 18519 & 18520, *Se*YCL012C with 18990 & 18991, *Sc*YCR051W with 18529 & 18530, *Sc*YCR087C-A with 18534 & 18535, *Se*Site1 with 18539 & 18540, *Se*Site2 with 18544 & 18545, *Se*Site3 with 18549 & 18550, *Se*Site4 with 18992 & 18993, *ScEEB1* with 15944 & 15945 and *ScEAT1* with 15950 & 15951.

Flow cytometry and cell sorting

After the transformation procedure, the cell suspension was plated onto selective medium (1/5th of the cells) and transferred to 20 mL liquid selective medium in vented tubes (4/5th of the cells) and cultivated for 5 days at 20 °C while shaking at 200 rpm. Then, 1 mL of the cell suspension was transferred to 20 mL fresh YPD hygR medium in shake flasks and grown for three days. Finally, 1 mL cell suspension was transferred to non-selective YPD medium for optimal fluorescent gene expression. *In vivo* assembly efficiency of ymNeongreen and ymScarletI genes in CBS 1483 was analysed by measuring fluorescence levels in the BD FACSAria™ II SORP Cell Sorter (BD Biosciences, NJ) equipped with 355, 445, 488, 561 and 640 nm lasers and a 70 µm nozzle, and operated with filtered FACSFlow™ software (BD Biosciences). The fluorophore ymScarletI was excited by the 561 nm laser and emission was detected through a 582 nm bandpass filter with a bandwidth of 15 nm. The fluorophore ymNeongreen was excited by the 488 nm laser and emission was detected through a 545 nm bandpass filter with a bandwidth of 30 nm. The cytometer performance was evaluated prior to each experiment by running a CST cycle with CS&T Beads (BD Biosciences) and the drop delay for sorting was determined by running an Auto Drop Delay cycle with Accudrop Beads (BD Biosciences). For each sample, 100.000 events were analysed. Cell morphology was analysed by plotting forward scatter (FSC) against side scatter (SSC) and the appropriate cell size was gated. Gated cells were used to determine the fluorescence intensity of the cells. Gating windows for fluorescence intensity were based on the fluorescence of the cells transformed with solely ymNeongreen or ymScarletI as repair fragment for sole integrations. Cells in the gate ymNeongreen^{+}ymScarletI^{+} were sorted separately on non-selective YPD plates and grown for five days. FACS data was analysed using the Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Finland).

Fermentation in septum flasks

Frozen aliquots of CBS 1483, IMI504-IMI508, IMI510-512, IMI483 and IMI485 were inoculated in 20 mL of YPD media in 50 mL Greiner tubes with air vents at 12 °C shaking at 200 rpm. Wake-up cultures were transferred to precultures in 100 mL YPD in 500 mL shake flasks grown at 12 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 100 mL septum bottles containing 60 mL full malt wort at 5.7 °P for strains CBS 1483, IMI504-IMI508, IMI510-512 or in full malt wort at 17 °P for strains CBS 1483, IMI483 and IMI485 at a starting OD_{660} of 0.2 mL^{-1}. The cultures were incubated at 12 °C and at 200 rpm for five (5.7 °P) or eight days (17 °P) with regular daily sampling to determine cell density, sugar consumption, ethanol production, esters and ketones concentrations throughout the fermentations.

Analytical techniques

Sugars (glucose, fructose, maltose, maltotriose) and ethanol concentrations were measured using HPLC (Agilent Technologies, 1260 HPLC system) equipped with a Bio-Rad HPX-42A column (300 × 7.8 mm, 25 micron) (Bio-Rad) and an 1260 Refractive Index Detector. The column was operated at 75 °C, and the elution was performed using milliQ at 0.5 mL min^{-1} for 30 min. Compounds were measured using a RID at 35 °C. Samples were 0.2 µm filter-sterilized before analysis.

Vicinal diketone (diacetyl, 2, 3-pentadione) concentrations were measured using static headspace gas chromatography in a 7890A Agilent GC (Agilent) with an electron capture detector on a CP-Sil 8 CB (50 m x 530 µm x 1 µm) capillary column. 0.5 mL of each sample was heated up to 65 °C for 30 min prior to injection. A split flow of 8 mL N_2 min^{-1} was used, with a split ratio of 1:1. The injector temperature was set at 120 °C. The oven was set at 35 °C for 3 minutes, followed by a 10 °C min^{-1} ramp to 95 °C. The ECD temperature was set at 150 °C with a make-up flow of 30 ml min^{-1} N_2.

Higher alcohols and esters were analyzed using static headspace gas chromatography (GC) with a flame ionization detector FID (Agilent technologies 7890A) and a DB-WAXetr capillary column (30 m x 320 µm x 1 µm). 2.5 mL samples were heated up to 50 °C for 5 min using a CTC Combi Pal headspace auto-injector prior to the injection. A split flow of 9.88 mL N_2 min^{-1} was used, with a split ratio of 5:8:1. The temperature of the injector was 250 °C, with a flow of 10 mL N_2 min^{-1}. The oven temperature profile started at 55 °C followed by an increase of 20 °C min^{-1} to 160 °C with a hold time of 4.75 min. The FID temperature was set at 250 °C with a make-up flow of 10 ml min^{-1} N_2.

Whole genome sequencing

Yeast genomic DNA of transformants was isolated using QIAGEN Genomic-tip 100/G kit (Qiagen). Genomic DNA concentrations were measured with the BR dsDNA kit (Invitrogen, Carlsbad, CA) using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

In-house DNA sequencing was performed using MiSeq System (Illumina, San Diego, CA). Extracted DNA was mechanically sheared to an aimed average size of 550 bp with the M220 ultrasonicator (Covaris, Wolburn, MA). DNA libraries were prepared using the TruSeq DNA PCR-Free Library Preparation Kit (Illumina) according to the manufacturer's manual. Quantification of the libraries was done by qPCR using the KAPA Library Quantification Kit for Illumina platforms (Kapa Biosystems, Wilmington, MA) on a Rotor-Gene qPCR cycler (Qiagen). The library quality was verified on the TapeStation System 4150 (Agilent). All libraries were normalized to 2 nM before the multiplexing step.

All Illumina sequencing (Table S1) data are available at NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject accession number PRJNA911296 (study SRP412695). The raw Illumina reads (Table S1) were aligned against a chromosome-level reference genome of CBS 1483 (NCBI bioproject accession number PRJNA522669, https://www.ncbi.nlm.nih.gov/) (Salazar *et al.*, 2019) using the Burrows–Wheeler Alignment tool (BWA) (Li, 2013) and further process with SAMtools (Danecek *et al.*, 2021).

Identification of unique landing sites

Identification of unique landing sites on CHRIII of the CBS 1483 was performed using sequence available at NCBI (https://www.ncbi.nlm.nih.gov/) as bioproject accession numbers PRJNA522669 (Illumina) and PRJNA522669 (Nanopore). The coding sequences and additionally 800 bp upstream and 300 bp downstream were subtracted from the *Se*Sc*CHRIII sequence. These non-coding sequences of *Se*Sc*CHRIII were extracted with bedtools (getfasta option) (Quinlan & Hall, 2010). The remaining sequence was used to generate k-mers with k = 200 and k = 500 with the ara package (https://github.com/AbeelLab/ara). The resulting k-mers were aligned to the CBS 1483 genome using a MUMmer alignment (Marçais *et al.*, 2018) and eliminated upon a match other than its original location in *Se*Sc*CHRIII. Next, the unique regions were subjected to manual inspection for presence of other essential DNA sequences and discarded when present. Finally, manual screening of the unique landing sites for other essential DNA regions was performed. Genes with non-essential functions as described in the SGD database (https://www.yeastgenome.org/) were selected.

CHRIII configuration analysis

S. pastorianus genomes deposited at NCBI (Bioprojects PRJNA522928 (Langdon *et al.*, 2019), PRJDB4073 (Okuno *et al.*, 2016), PRJNA504476, PRJNA169496) were mapped to the concatenated genome sequences of *S. eubayanus* (CBS 12357) and *S. cerevisiae* (CEN.PK113-7D) using the Burrows-Wheeler Alignment (BWA) tool (Li, 2013) and further process with SAMtools (Danecek *et al.*, 2021). Coverage of 500 bp window was calculated across the reference and CNV was determined based on Magnolya (Nijkamp *et al.*, 2012).

4.3. Results

Targeting homoeologous genes in CBS 1483 results in unpredictable loss of heterozygosity

To illustrate the unpredictability of the outcome of CRISPR editing in allo-aneuploidy yeast, individual knockout of genes *ScEEB1* and *ScEAT1* was attempted. *ScEEB1* is carried by *Sc*CHRXVI (two copies) and has an homoeolog (*SeEEB1*) on *Se*CHRXVI (two copies). *ScEAT1* is present on *Sc*CHRVII (one copy) and its homoeolog *SeEAT1* is on *Se*CHRVII (three copies). Deletion of the two copies of *ScEEB1* in *S. pastorianus* CBS 1483 was performed by co-transforming pUDP168 that expresses a gRNA targeting *ScEEB1* and a repair fragment containing the ymNeongreen gene flanked by 60 bp nucleotides homologous to upstream and downstream the *ScEEB1* open reading frame. Similarly, deletion of *ScEAT1* was performed using pUDP172 and a ymNeongreen containing repair targeting the *ScEAT1* locus (Figure 4.1AB). Integration of ymNeongreen at the target site resulted in green fluorescent colonies when excited by blue light (Figure 4.1AB). Despite the previously shown successful editing of *SeILV6*, *SeATF1* and *SeATF2* in CBS 1483 with CRISPR-Cas9 (Gorter de Vries *et al.*, 2017), integration of the ymNeongreen marker targeting of *ScEEB1* or *ScEAT* yielded efficiencies of 0.19% and 0.48%, respectively (Figure 4.1C). Even though fluorescent transformants were observed, none of them showed the right genotype (Figure 4.1DE). To eliminate the possible weak activity of the gRNA to programme Cas9 targeting at both sites, the same two deletions were also performed in the haploid *S. cerevisiae* CEN.PK113-7D. Contrasting with the poor efficiency observed in CBS 1483, all CEN.PK113-7D fluorescent transformants tested were harbouring the correct deletion (Figure 4.1CFG).

Assuming that the Cas9 guided by the respective gRNAs introduced the DSB successfully, the remaining question is how does *S. pastorianus* repair the DSB event that, if not repaired, is lethal for the cell. To elucidate this mechanism, four non-fluorescent transformants from *ScEEB1* deletion transformation were randomly selected and subjected to whole genome sequencing.

Sequencing analysis confirmed that transformants had failed to incorporate the supplied repair fragment, but instead repaired the DSB with the homoeologous *S. eubayanus* CHRXVI as template for HDR resulting in loss of heterozygosity (Figure 4.2, Figure S1, Table S1). Each transformant exhibited a different pattern: colony 1 (IMK1062), colony 4 (IMK1064) and colony 3 (IMK1063) were repaired by a 5 kbp, 10 kbp and 26-kbp region surrounding the *ScEEB1* gene, respectively, while in colony 6 (IMK1065) over 700 kbp from the *Sc*CHRXVI was replaced by the homologous sequence from the *Se*CHRXVI sequence and only the subtelomeric regions of *Sc*CHRXVI were retained (Figure 4.2, Figure S1, Table S1). Therefore, loss of heterozygosity resulting from preferential usage of the homoeologous chromosome is an undesired side-effect

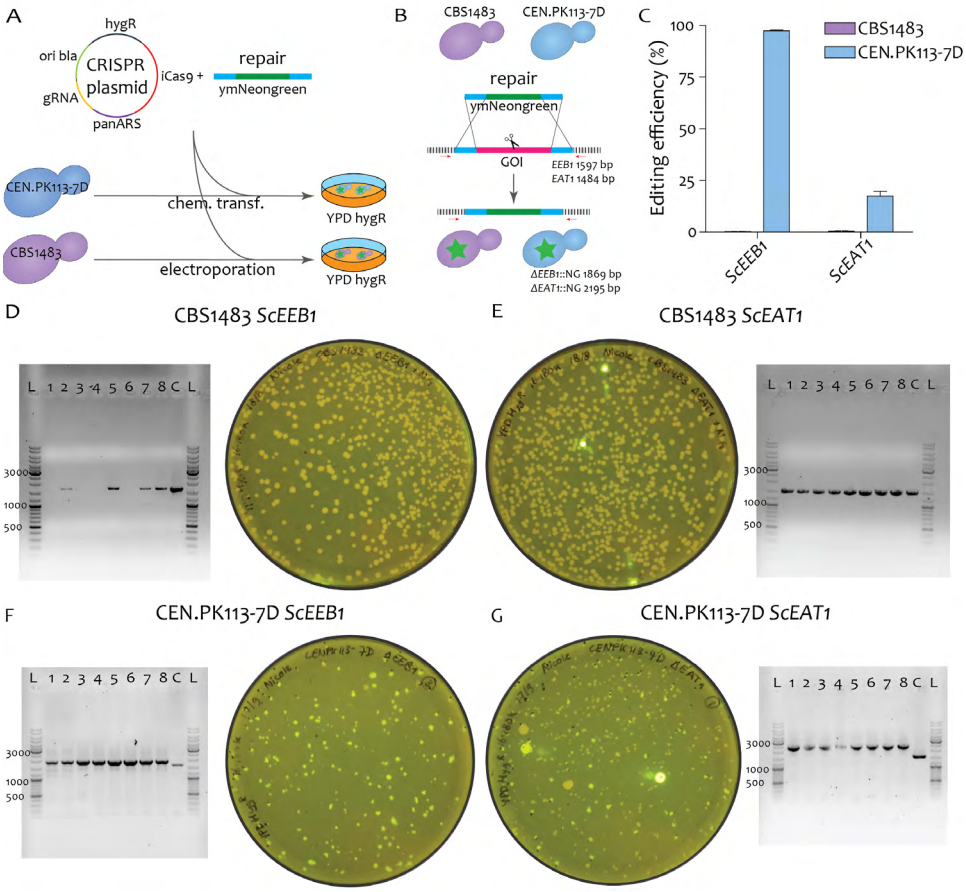


Figure 4.1. Genome editing in haploid *S. cerevisiae* CEN.PK113-7D and allo-aneuploid *S. pastorianus* CBS 1483. **A)** Schematic representation of the transformation setup and genomic modification. **B)** Genome editing efficiencies in *S. pastorianus* CBS 1483 (purple bars) and *S. cerevisiae* CEN.PK113-7D (blue bars) targeting the *S. cerevisiae* allele (CBS 1483) or gene (CEN.PK113-7D) *ScEEB1* and *ScEAT1* thereby aiming for ymNeongreen integration. Editing efficiencies were calculated based on number of green fluorescent colonies over the total number of colonies on the transformation plates. Values are derived from three individual transformations. Transformation plates and genotyping of transformants of ymNeongreen integration in *ScEEB1* in *S. pastorianus* CBS 1483 **(C)**, *ScEEB1* in *S. cerevisiae* CEN.PK113-7D **(D)**, *ScEAT1* in *S. pastorianus* CBS 1483 **(E)** and *ScEAT1* in *S. cerevisiae* CEN.PK113-7D **(F)**. Integration of ymNeongreen results in green fluorescent colonies. Genotyping confirmed integration of ymNeongreen in *ScEEB1* and *ScEAT1* in *S. cerevisiae* CEN.PK113-7D, but not in *S. pastorianus* CBS 1483.

of Cas9-mediated genome editing and has major impact for the editing specificity and efficiency in allo-aneuploid yeasts, including *S. pastorianus* CBS 1483.

A systematic strategy to identify unique landing sites for CRISPR-Cas9 targeting

Genome editing with CRISPR-Cas9 allows to make gene deletions, gene integrations and gene modifications. In the case of gene deletions and gene modifications, the user

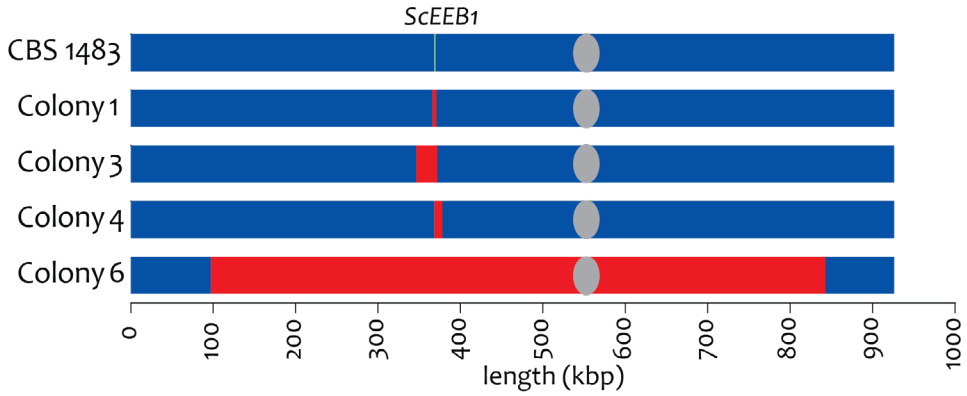


Figure 4.2. Whole genome sequencing reveals that CRISPR-Cas9 editing in the allo-aneuploid yeast *S. pastorianus* CBS 1483 aiming to integrate ymNeongreen in *ScEEB1* on ScCHXVI results in loss of heterozygosity in all four colonies tested. The red and blue color indicate chromosome assigned as *S. eubayanus* and *S. cerevisiae*, respectively.

is restricted to the location of the gene of interest in the genome. On the other hand, additional genes can be integrated at any user-chosen genomic location. Therefore, identification of chromosomal regions (landing sites) devoid of homoeologous regions for gene integrations with CRISPR-Cas9 in the allo-aneuploid yeast strain *S. pastorianus* CBS 1483 would be of a prime importance. Landing sites should meet the following criteria: i) the frequency for LOH is low, ii) the gRNA targets the protospacer with high efficiency and iii) gene integration does not result in undesired changes in the cell's physiology. To prevent unwanted repairs that would use the homoeologous chromosome, the search for landing sites was first limited to the chimeric *Se*ScCHRIII that is composed for two-third from the *S. eubayanus* parent and one-third from *S. cerevisiae* (van den Broek *et al.*, 2015, Salazar *et al.*, 2019). First, a systematic approach to find suitable integration sites on *Se*ScCHRIII was developed. Since modification at a landing site should remain metabolically neutral, in other words should not impact yeast physiology, coding sequences including 800 bp upstream and 300 bp downstream covering the promoter and terminator regions were subtracted from the *Se*ScCHRIII sequence. This *in silico* thorough analysis revealed a set of 23 non-coding regions (total of 34.7 kbp) ranging from 30 bp to more than 6 kbp (Figure 4.3, Table S2). These non-coding sequences were used to generate k-mers of 100, 150, 200, 300 and 500 nucleotides which were aligned to CBS 1483 genome using MUMmer (Marçais *et al.*, 2018). K-mers with small, partial alignments to other chromosomes were discarded to avoid selection of sequences containing homology with other parts of the genome and only k-mers that returned one hit located on *Se*ScCHRIII were selected. This resulted in a total of 283 and 50 unique k-mers for respectively k = 200 and k = 500, residing in a total of 26 regions (total 22.4 kbp, average length of 863 bp) and 17 regions (total 23.9 kbp, average length of 1403 bp), respectively. Since unique k-mers could be adjacent and overlap to one another, the 17 unique regions of the k = 500 output were identified

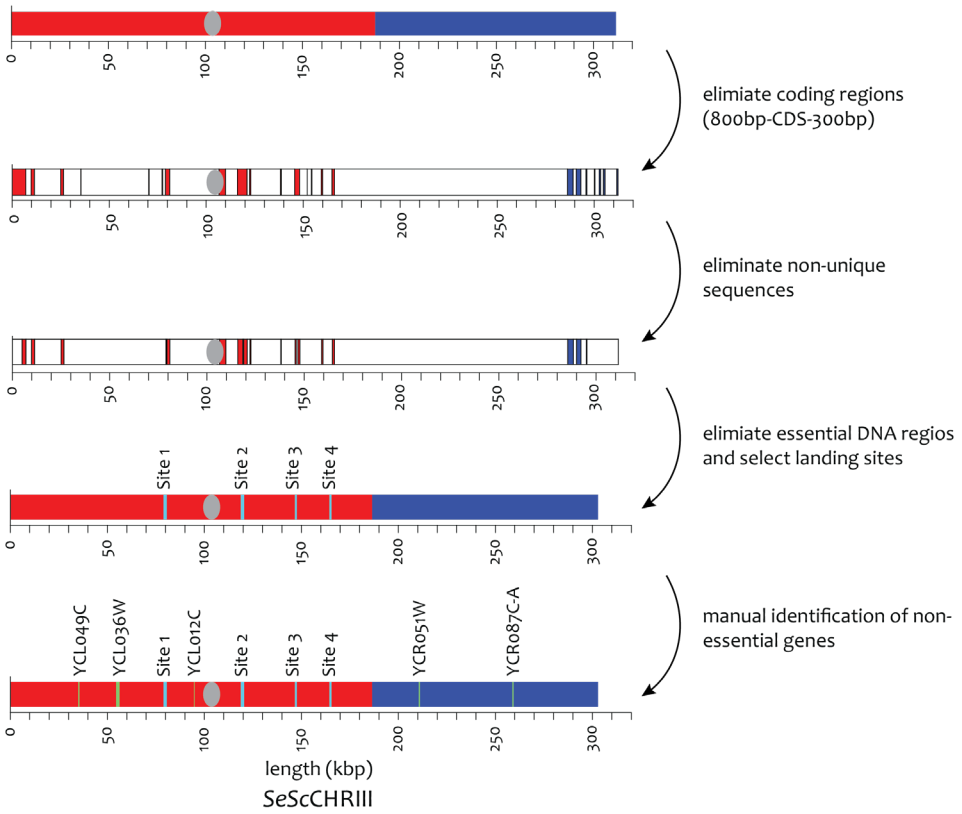


Figure 4.3. Strategy to identify non-coding unique DNA sequences in *S. pastorianus* CBS 1483 *Se*Sc*CHRIII*. In the first step, non-coding regions were obtained by subtracting the coding sequences plus 800 bp upstream and 300 bp downstream covering for promoter and terminator regions. In the second step, the unique regions were obtained by selecting unique *k*-mers when aligning to the CBS 1483 genome and the non-unique sequences were eliminated. In the next step, the non-coding unique sequences were checked for the presence of essential DNA regions. Additionally, potential landing sites identified by manual screening of non-essential genes were selected, resulting in a total of nine landing sites.

spanning lengths from 198 to 3213 bp. These 17 unique regions were manually checked for presence of other essential DNA regions, such as tRNAs, telomeres, centromeres and autonomous replication sequences. Out of the 13 regions that passed the final check, four landing sites distributed over *Se*Sc*CHRIII* referred to as Site 1, 2, 3 and 4, were chosen to investigate their potential use for genomic integrations in CBS 1483. Secondly, a manual screening of *Se*Sc*CHRIII* for non-essential genes was deployed to find landing sites located in gene encoding regions. The search resulted in 13 genes suitable as landing sites (Table S3) of which the following five genes distributed over the chromosome were selected: *Se*YCL049C, *Se*YCL036W, *Se*YCL012C, *Sc*YCR051W and *Sc*YCR087C-A. In total, nine genes and sites were retained as potential landing sites on CBS 1483 *Se*Sc*CHRIII* (Table 4.4, Figure 4.3).

Table 4.4. Overview of the selected landing sites on *Se*ScCHRIII in *S. pastorianus* CBS 1483.

Landing site	Start coordinate	Stop coordinate	Strand	Size (bp)
*Se*YCL049C	35643	35915	-	884
*Se*YCL036W	54552	56237	+	1685
*Se*Site 1	79419	80823	n.a.	1404
*Se*YCL012C	93151	93617	-	466
*Se*Site 2	118822	120628	n.a.	1806
*Se*Site 3	146704	147799	n.a.	1095
*Se*Site 4	164540	165542	n.a.	1002
*Sc*YCR051W	209678	210346	+	668
*Sc*YCR087C-A	259551	259985	-	434

n.a. Not Applicable

CRISPR-Cas9 targeting of unique landing sites allows efficient gene integration

To evaluate the landing sites for genomic integrations, gRNAs for all landing sites were designed considering GC-content, secondary structure, off-targets and allelic variation that may occur within the four copies of the CBS 1483 chimeric *Se*ScCHRIII (Table S4). To evaluate the CRISPR-editing in the nine selected landing sites, the CRISPR-plasmid co-expressing *Streptococcus pyogenes* iCas9^{D147Y,P411T} and the respective gRNA (Juergens *et al.*, 2018) were co-transformed with the ymNeongreen repair fragment, allowing visual screening of edited colonies, into CBS 1483.

Targeting the landing sites *Se*YLC049C, *Se*YCL036W, *Se*YCL012C, *Sc*YCR051W, *Sc*YCR087C-A, *Se*Site2, *Se*Site3 and *Se*Site4 resulted in high editing efficiencies, which is reflected by the number of green fluorescent colonies on the transformation plates (Figure 4.4). Genotyping of ten colonies for each landing sites showed 100% correct ymNeongreen integration in *Se*YCL049C, *Se*YCL012C, *Sc*YCR051W, *Sc*YCR087C-A, *Se*Site2, *Se*Site3 and *Se*Site4 (Figure S2). Genotyping of *Se*YCL036W showed that eleven out of the twelve assessed colonies were correctly edited and showed correct integration of the ymNeongreen expression cassette (Figure S3).

Only integration at *Se*Site1 did not result in high editing efficiencies, possibly due to the low gRNA efficiency (Figure 4.4). Therefore, eight out of nine identified landing sites showed high potential for standard genomic integrations in *S. pastorianus* CBS 1483 as they were targeted with high efficiency and do not cause loss of heterozygosity.

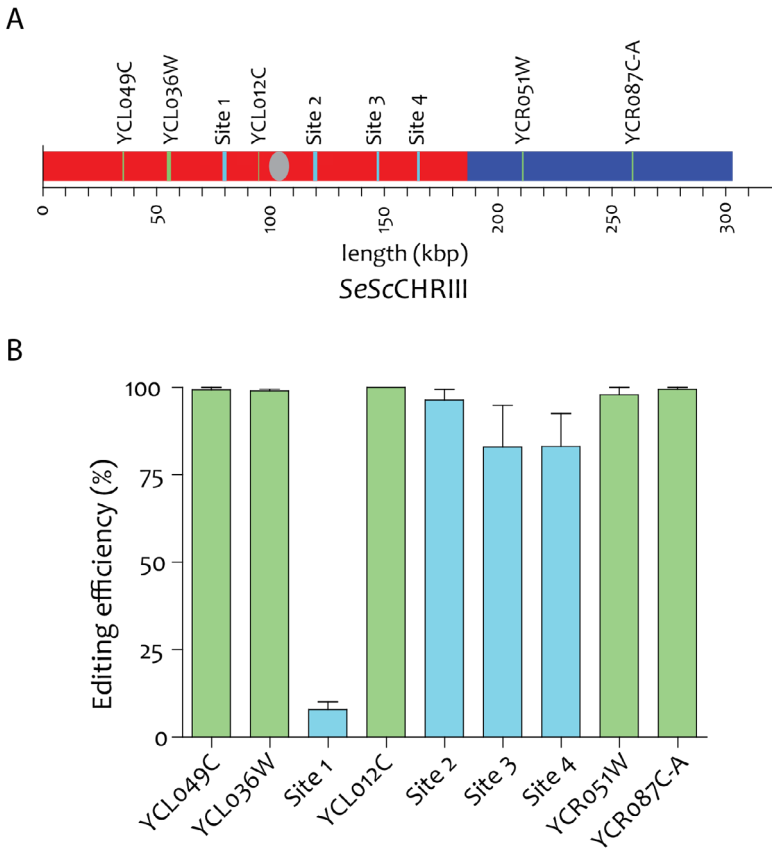


Figure 4.4. A) Overview of the identified landing sites in CBS 1483. **B)** Genome editing efficiencies in *S. pastorianus* CBS 1483 targeting the identified landing sites on *Se*Sc*CHRIII* thereby aiming for ymNeongreen integration. Editing efficiencies were calculated based on number of green fluorescent colonies over the total number of colonies on the transformation plates. Values are derived from three individual transformations.

Some of the landing sites can be used in multiple *S. pastorianus* strains: the example of Weihenstephan 34/70

The use of these integration sites is not just restricted to the CBS 1483 strain. Genome-wide analysis of different *S. pastorianus* genomes deposited at NCBI (https://www.ncbi.nlm.nih.gov/), revealed that the large majority of the strains (45 out of 47 strains) harbours the chimeric *Se*Sc*CHRIII* in various copy numbers, either as sole CHRIII configuration (two strains) or in combination with *Sc*CHRIII (39 strains) or *Se*CHRIII (four strains) (Figure 4.5A). In any configuration, the entire *Se*Sc*CHRIII*, if present alone as in CBS 1483, or part of it is unique. This is exemplified by the *S. pastorianus* WS 34/70 strain, which counts three copies of the chimeric *Se*Sc*CHRIII* and one full length copy of the *Sc*CHRIII (Figure 4.5B). Whereas the sequence similarity of the shared *Sc*CHRIII part is close to 100%, the sequence homology between the *Se*CHRIII part and the *Sc*CHRIII

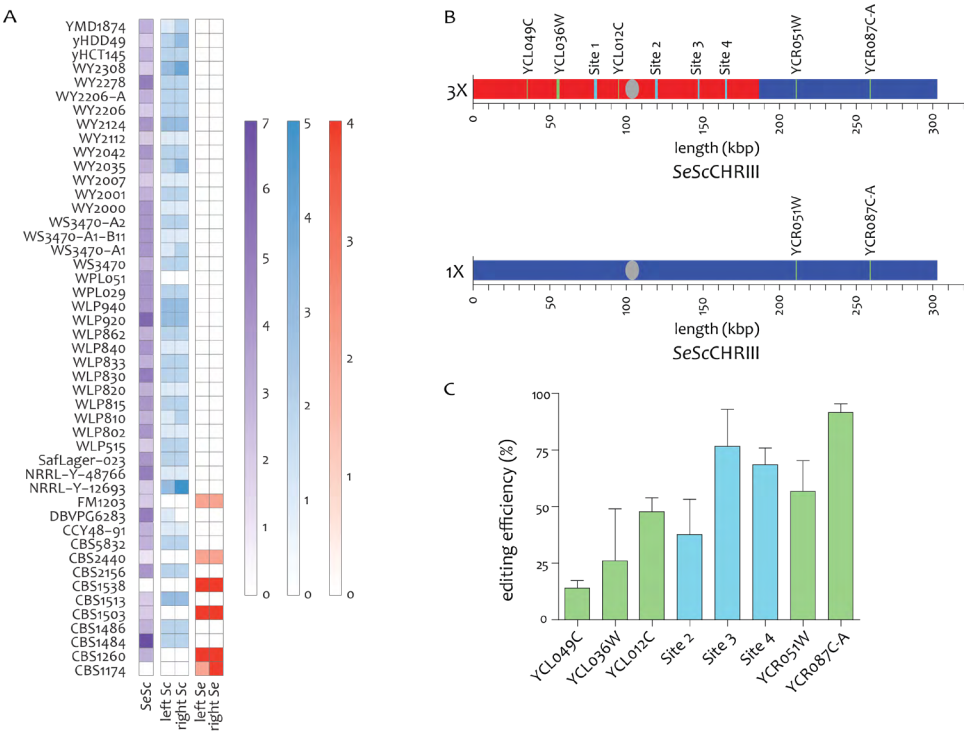


Figure 4.5. A) Overview of CHRIII genetic configurations in 47 different *S. pastorianus* strains. **B)** Chromosome III configurations in *S. pastorianus* WS 34/70. In comparison to CBS 1483, WS 34/70 has an additional *Sc*CHRIII and one less hybrid *Se*ScCHRIII. **C)** Genome editing efficiencies in *S. pastorianus* WS 34/70 targeting the identified landing sites thereby aiming for ymNeongreen integration. Editing efficiencies were calculated based on number of green fluorescent colonies over the total number of colonies on the transformation plates. Values are derived from three individual transformations.

part is only 80-85%. In this genome configuration it means that only the distal *S. cerevisiae* sequence of *Se*ScCHRIII including the *Sc*YCR051W and *Sc*YCR087C-A sites could lead to efficient editing. To test this hypothesis, the WS 34/70 strain was transformed with the gRNA expressing plasmids targeting the eight most efficient landing sites previously identified and their respective repair DNA containing the fluorescent marker. The integration sites lacking homoeologous sequences, *Sc*YCR051W or *Sc*YCR087C-A, showed as anticipated a high targeting efficiency exceeding 50% (Figure 4.5B). For the proximal landing sites *Se*YCL049C, *Se*YCL036W, *Se*YCL012C and *Se*Site2 there was a significant drop in efficiency (<50%). Remarkably, the efficiency of the editing at *Se*Site3 and *Se*Site4 was to a lesser extent not hampered by the presence of the *S. cerevisiae* sequence contrasting with results at other chromosomal sites indicating as well that other genetic factors might play a role.

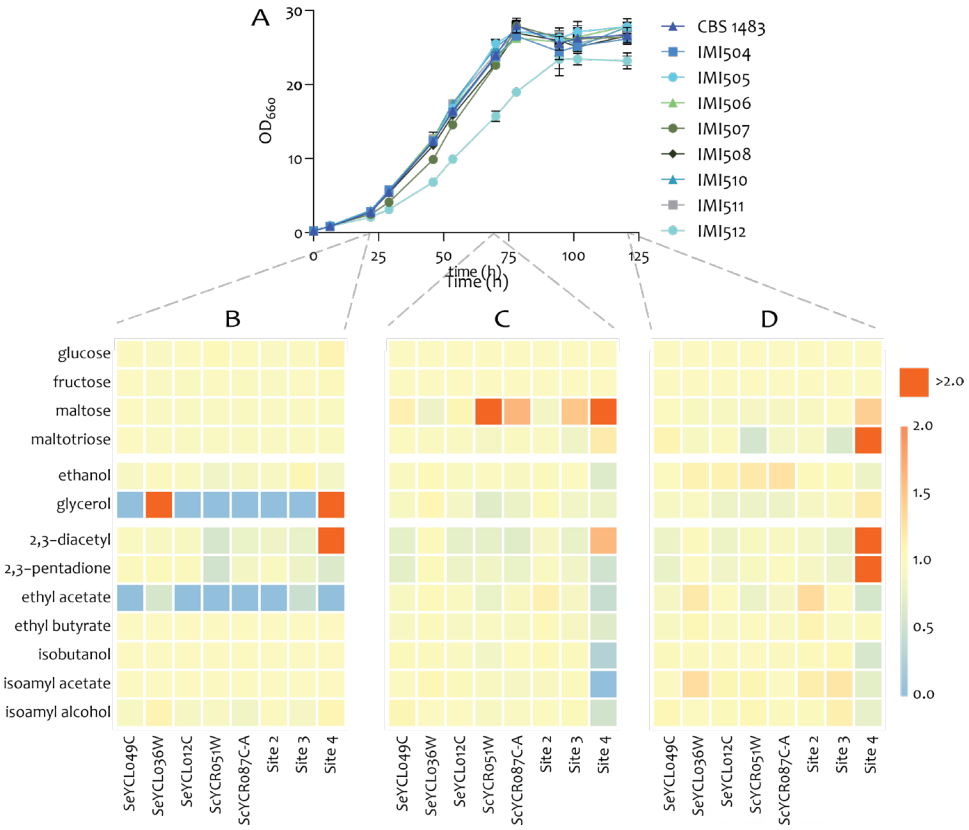


Figure 4.6. Physiological characterization of landing site disruptions under brewing conditions. **A)** Growth curves as function of OD_{660} nm of the S. *pastorianus* strain CBS 1483 and derived strains IMI504 (Δ*Se*YCL049C), IMI505 (Δ*Se*YCL036W), IMI506 (Δ*Se*YCL012C), IMI507 (Δ*Sc*YCR051W), IMI508 (Δ*Sc*YCR087C-A), IMI509 (Δ*Se*Site1), IMI510 (Δ*Se*Site2), IMI511 (Δ*Se*Site3), IMI512 (Δ*Se*Site4) harboring a ymNeongreen expression cassettes inserted at single landing sites grown in septum flasks under brewing conditions with full malt wort (5.7° P) at 12 °C. The values represent averages ± mean deviations of data obtained from independent triplicate cultures. At **B)** t19, **C)** t68 and **D)** t116 hours supernatant samples were analysed for extracellular sugars (glucose, fructose, maltose, maltotriose), ethanol, glycerol, vicinal diketones (diacetyl, 2,3-pentadione), higher alcohols (isoamyl alcohol, isobutanol) and ethyl (ethyl acetate, ethyl butyrate) and acetate (isoamyl acetate) esters. Concentrations relative to the reference strain CBS 1483 are represented in heatmaps and fold-change in concentrations are color-coded according the scale provided right to heatmap **D)**.

Genomic integrations at landing sites do not result in changes in physiology and brewing characteristics

One of the criteria for a good landing site is that integration of DNA at this location should not have a physiological effect on the cell in comparison to the parental CBS 1483 strain. To investigate potential physiological effect on genomic integrations, the strains CBS 1483 and IMI504-8 and IMI510-512 containing ymNeongreen in their

respective landing site, were cultivated in septum flasks under brewing conditions (full malt wort at 5.7 °P and at 12 °C). Deviations in growth, sugar consumption, flavour molecule production and ethanol production were evaluated relative to the parental strain CBS 1483 (Figure 4.6). Early in the fermentation, the deviations in produced metabolites (e.g. glycerol (0.013 ± 0.033 g L^{-1} for CBS 1483 and 0.000 ± 0.000 g L^{-1} for all other strains, compared to > 0.700 g L^{-1} respective end concentrations) and ethyl acetate (0.147 ± 0.127 mg L^{-1} for CBS 1483 and 0.000 ± 0.000 mg L^{-1} for six other strains, compared to > 1.100 mg L^{-1} respective end concentrations) can have large effects on the fold change, but represent only a very small fraction of the final concentrations. Integration in the landing sites *Se*YCL049C, *Se*YCL036W, *Se*YCL012C, *Sc*YCR051W, *Sc*YCR087C-A, *Se*Site2 and *Se*Site3 did not have a significant effect on the physiological behaviour of the strain and are therefore suited for gene integrations. In contrast, IMI512 (*Se*Site4::ymNeongreen) showed slower growth as well as sugar consumption and ethanol production compared to the parental strain CBS 1483. Also, the production of vicinal diketones and esters that contribute to beer flavour was lower for ethyl acetate, isoamyl alcohol, isoamyl ester and isobutanol, whereas the production of ethyl butyrate was higher compared to CBS 1483 after 6 days of cultivation. These observations demonstrated that *Se*Site4 was less suitable for integration of genes of interest.

Integration of acetolactate decarboxylase to eliminate diacetyl production

To demonstrate the reliability of the selected sites, lager brewing yeast strains with reduced diacetyl production were constructed. The vicinal diketone diacetyl is one of the main off-flavours in lager-style beers. The lagering period after the fermentation is required for the reduction of diacetyl and has a major impact on the time and energy of the brewing process. Diacetyl is formed in the supernatant by spontaneous decarboxylation from excreted α-acetolactate, an intermediate of valine and leucine biosynthesis. During the lagering, the brewing yeast reduces diacetyl sequentially to acetoin and 2,3 butanediol (Godtfredsen & Ottesen, 1982, Bamforth & Kanauchi, 2004).

To prevent α-acetolactate leakage, an α-acetolactate decarboxylase (*aldC*) that catalyzes the decarboxylation of α-acetolactate to acetoin can be expressed. *α-aldC* genes from prokaryotic origin have already been successfully expressed in brewing yeasts, including *S. cerevisiae* (Sone *et al.*, 1988, Blomqvist *et al.*, 1991), *S. uvarum* (Sone *et al.*, 1988, Yamano *et al.*, 1995) and *S. carlsbergensis* (Fujii *et al.*, 1990) and *S. pastorianus* (Suihko *et al.*, 1990) and have shown great reduction in diacetyl formation resulting in significant shortening of the lagering. We chose two different α-aldC genes originating from *Brevibacillus brevis* (*BbaldC*) and *Leuconostoc lactis* (*LlaldC*). To ensure high gene expression levels, the genes were placed under control of the constitutive *ScPGK1* promoter (Knijnenburg *et al.*, 2009) and the *ScADH1* terminator. Linear fragments containing the expression cassettes with 60 bp homologous flanks

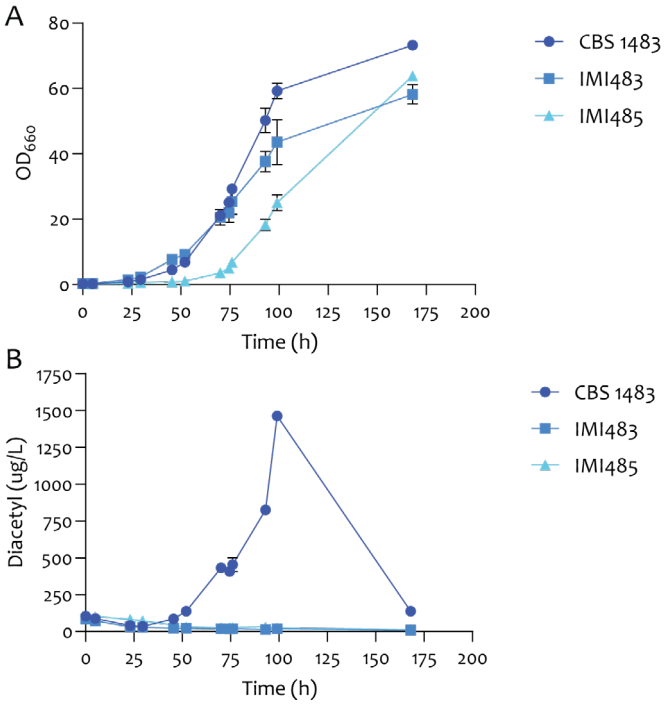


Figure 4.7. Performance of CBS 1483 strains expressing different prokaryotic *aldC* genes. **A)** Growth curve and **B)** diacetyl concentration of CBS 1483, IMI483 (expressing *BbaldC*) and IMI485 (expressing *LlaldC*) grown on full malt wort (17 °P, 12 °C). The values represent averages ± mean deviations of data obtained from independent triplicate cultures.

were co-transformed with the CRISPR-plasmid pUDP273 targeting the landing site ScYCR087C-A into CBS 1483. The transformation efficiency, calculated based on the percentage of positive colonies verified by diagnostic PCR, was 100% and 79% for *BbaldC* and *LlaldC*, respectively (Table S6). The correctly edited transformants were named IMI483 (*BbaldC*) and IMI485 (*LlaldC*).

The strains IMI483 (*BbaldC*) and IMI485 (*LlaldC*), were assessed for diacetyl production in septum flask cultivation on 17 °P full malt wort at 12 °C and compared to CBS 1483. In agreement with previous results (Godtfredsen & Ottesen, 1982, Godtfredsen *et al.*, 1984, Sone *et al.*, 1988, Fujii *et al.*, 1990, Blomqvist *et al.*, 1991, Yamano *et al.*, 1994, Yamano *et al.*, 1995, Guo *et al.*, 2001), heterologous expression of an *α-aldC* gene in CBS 1483 likely eliminated the diffusion of α-acetolactate into the extracellular space preventing formation of diacetyl and therefore the need of its reduction (Figure 4.7). Nonetheless, the growth of IMI485 was hampered compared to the parental strain CBS 1483, caused by loss of the ability to consume maltotriose (Figure S4). This case showed that the landing sites identified for *S. pastorianus* CBS 1483 were suitable for efficient and reliable engineering requiring CRISPR-Cas9 guided integration.

Successful genomic integration through *in vivo* assembly into *Sc*/*Se*CHRIII landing sites

One of the startling genetic characteristics of *S. cerevisiae* is its ability to recombine multiple linear DNA fragments at a single chromosomal locus. This is often referred to as *in vivo* DNA assembly. We demonstrated that elimination of competing mechanisms using the homoeologous chromosome as template improved targeting efficiency of repair fragment integration at chosen chromosomal sites. To investigate the possibility to integrate two fragments simultaneously by homology directed repair, *S. pastorianus* CBS 1483 was co-transformed with pUDP269, plasmid that expresses a gRNA targeting the *Se*YCL049C landing site and two DNA repair fragments. The first DNA fragment consisted of the ymNeongreen expression cassette that was flanked by 60 bp homologous flanks upstream to the *Se*YCL049C genomic locus and 60 bp SHR sequences (Kuijpers *et al.*, 2013), whereas the second fragment encoded the ymScarletI gene flanked by 60 bp SHR sequence complementary to that of the first fragment and 60 bp homologous flanks downstream the *Se*YCL049C landing site. The use of fluorescent genes for the integration allows for screening of the transformed cell population by Fluorescence-activated cell sorting. Screening of the transformant population revealed that 17.67 ± 1.98 % of the transformed cells harboured both fluorescent markers (Figure S5). To confirm that the fluorescence was derived from single and not aggregated cells, single cells from the ymNeongreen^{+} ymScarletI^{+} gated population were sorted on non-selective medium (Figure S5). Cultivation of the sorted single cells and subsequent flow cytometry analysis of 12 clones confirmed co-occurrence of both ymNeongreen and ymScarletI fluorescence (Figure S6). Genotyping revealed in all 12 screened colonies correct integration of both genes in the *Se*YCL049C landing site (Figure S8). These results showed that genomic integration of *in vivo* assembled DNA fragments is also possible in the interspecific hybrid *S. pastorianus*.

4.4. Discussion

CRISPR-mediated gene editing in diploid and more generally polyploid heterozygous *S. cerevisiae* is drastically impaired when not all chromosomes are simultaneously targeted by a programmed CRISPR endonuclease 9 (Gorter de Vries *et al.*, 2018). In such conditions, the still intact homologous chromosome is used as preferential template to fix the double strand break by homology-directed repair. This resolution of the introduced cut can result in chromosome recombinations, which can lead to loss of heterozygosity (Ma *et al.*, 2017, Gorter de Vries *et al.*, 2018, Lombardi *et al.*, 2022). LOH competes with an intended gene-editing repair event and results in reduced editing efficiencies and possibly in extensive genetic changes. In this study, we demonstrated that this mechanism could also occur between homoeologous chromosomes in the interspecific hybrid yeast *S. pastorianus*. While in diploid yeasts the problem might be restricted to few heterozygous locations and easily solved by designing guide-RNA targeting all chromosomes, in an interspecific hybrid that retained the quasi complete

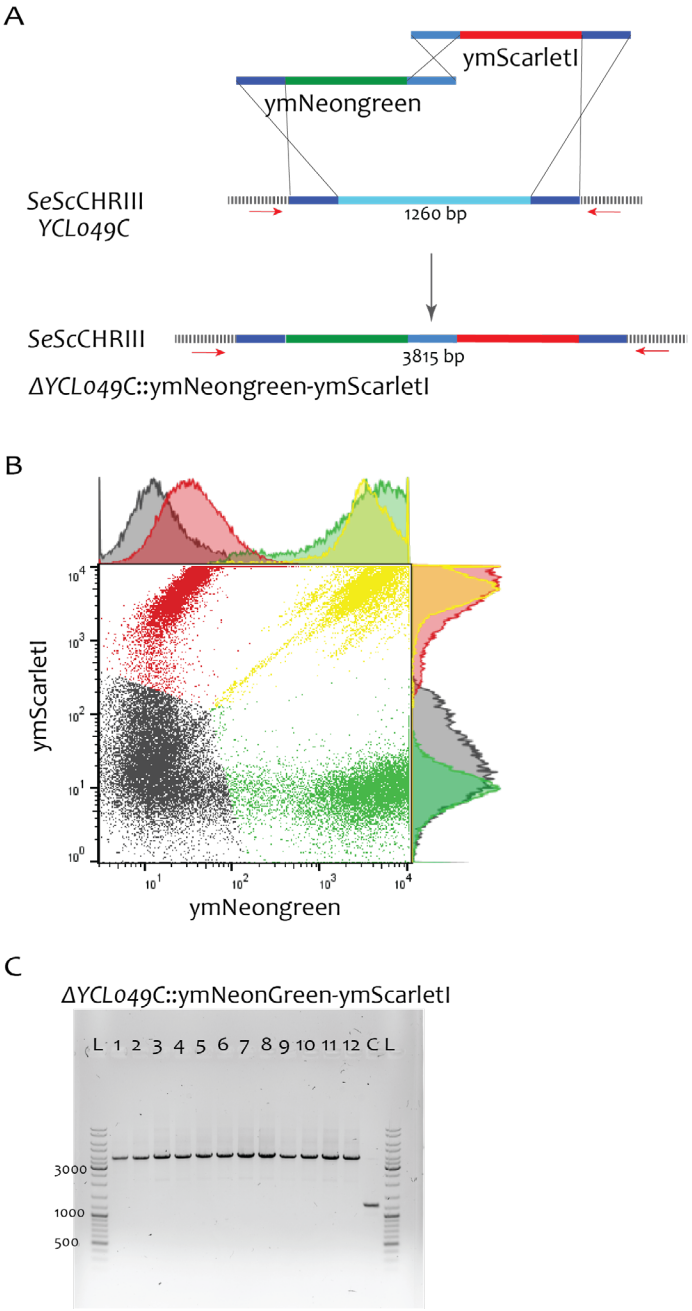


Figure 4.8. Genomic integration into landing site *Se*YCL049C of two *in vivo* assembled DNA fragments expressing ymNeongreen and ymScarletI. **A)** Schematic overview of the genomic integration strategy for *in vivo* assembly of ymNeongreen and ymScarletI DNA fragments. **B)** Fluorescent profile of the transformed population when aiming for integration of two fluorescent expression cassettes. The fluorescence corresponding to ymNeongreen and ymScarletI is plotted for 100.000 events. Flow cytometry was performed for three biological replicates; one representative replicate is shown. **C)** Genotyping of the sorted ymNeongreen^{+}ymScarletI^{+} single colony isolates.

parental genomes, the problem is extended to every single nucleotide since any edit on the chromosome of one of the sub-genome e.g. *S. cerevisiae*, can be repaired with its homoeologous counterpart e.g. *S. eubayanus*. As observed in this study, whether repaired by the donor repair DNA or by the homoeologous chromosome, the resulting chromosomal locus was converted identically in all copies suggesting that the first repaired site preferentially converts the other. This unpredictability unfortunately affects the progress of functional genetic studies in hybrid, industrial yeasts since introduction of accurate genetic alterations would have to rely on HDR without introduction of a DSB (Baudin *et al.*, 1993, Wach *et al.*, 1994), methods that require the use of selectable markers and are iterative in nature since it can replace one allele at the time (e.g. deletion of a gene carried by a trisomic chromosome will require three rounds of transformations) (Duong *et al.*, 2011, Bolat *et al.*, 2013). Therefore, improving the predictability of the editing event is critical to enable efficient gene deletion or *in vivo* site-directed mutagenesis.

Genome-wide analysis of different *S. pastorianus* genomes deposited at NCBI (https://www.ncbi.nlm.nih.gov/) showed that chimeric *Se*Sc*CHRIII chromosome was nearly always present (found present in 45 out of the 47 *S. pastorianus* genomes analysed (Figure 4.5A)) as sole CHRIII or in combination with either a *Se*CHRII or *Sc*CHRIII version. In any configuration, the entire CHRIII or part of it is unique. In contrast to targeted sites on other chromosomes, the targeting efficiency of the site on *Se*Sc*CHRIII region devoid of homoeologous sequences was high and reached up to 100% in some instances. Refuting previous statements, these results demonstrated that homologous recombination was not impaired in *S. pastorianus* but was instead masked. We could also exclude that genome complexity, increased ploidy and chromosome copy number was hindering efficient repair of DSB, as *S. pastorianus* CBS 1483 could efficiently repair tetrasomic *Se*Sc*CHRIII. CRISPR-Cas9 mediated *in vivo* recombination of two DNA fragments further confirmed that at selected loci *S. pastorianus* could perform elaborated homology directed repair with high efficiency. Improving the targeting efficiency in lager brewing yeasts, provides new opportunities to accurately and time-effectively engineer *S. pastorianus* strains with new characteristics. Such strategies could swiftly and efficiently improve a wide range of phenotypic traits and fermentation characteristics, including wort sugar utilization, fermentation rate and energetic performance (Ekberg *et al.*, 2013, Brickwedde *et al.*, 2017), reduction of off-flavor (Suihko *et al.*, 1990, Duong *et al.*, 2011) and balanced flavour profiles, and, moreover, engineering of novel flavors (Hansen *et al.*, 2009, Denby *et al.*, 2018).

However, it remains important to evaluate the targeted integration on the genetic level and screen multiple colonies on phenotype to avoid loss of relevant characteristics resulting from secondary effects. It is indeed known that transformation procedures are mutagenic and in such an allo-aneuploid organism may lead to chromosome copy number variations that could impact other phenotypic traits unintentionally (van den

Broek *et al.*, 2015, Gorter de Vries *et al.*, 2020). To completely eliminate this possibility, systematic genome sequencing of engineered strains could be implemented.

Even though *S. pastorianus* is the most industrially used interspecific hybrid between *Saccharomyces* species yeasts, numerous independently formed natural hybrids between *Saccharomyces* species (e.g. *S. cerevisiae* × *S. kudriavzevii*, *S. cerevisiae* × *S. eubayanus* and *S. cerevisiae* × *S. kudriavzevii* × *S. eubayanus*) have been isolated from wine, beer, or cider fermentations (Hawthorne & Philippsen, 1994, Masneuf *et al.*, 1998, Le Jeune *et al.*, 2007, Arroyo-López *et al.*, 2009, Gangl *et al.*, 2009, Borneman *et al.*, 2012). Natural interspecific hybrids are not limited to the *Saccharomyces* species group. Several examples have also been described in *Zygosaccharomyces* (Ortiz-Merino *et al.*, 2017) and *Millerozyma* (Louis *et al.*, 2012). Often, these hybrids exhibit better characteristics in fermentation processes than the parental strains, as this heterosis provides a competitive advantage by enabling transgressive phenotypes in man-made environments, and also drives adaptation and fungal evolution. The guiding principles presented in our study should also be helpful to dissect the genetic background of heterosis in yeast hybrids other than *S. pastorianus* and contribute to engineer these yeasts in the future.

Data availability

Supplementary information can be accessed via the online publication.

Acknowledgments

We thank Sofia Dashko for cloning plasmid pGGKd034 and Sasha Yogiswara for cloning pUDP168 and pUDP172. We are thankful to Prof. J.T. Pronk (Delft University of Technology), Dr. V.M. Boer and Mr T. Elink Schuurman (HEINEKEN Supply Chain B.V.) for their support during this project.

4.5. References

Al-Attar S, Westra E, van der Oost J & Brouns S (2011) Clustered regularly interspaced short palindromic repeats (CRISPRs): the hallmark of an ingenious antiviral defense mechanism in prokaryotes. *Biological Chemistry* **392**: 277-289.

Arroyo-López FN, Orlić S, Querol A & Barrio E (2009) Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid. *International Journal of Food Microbiology* **131**: 120-127.

Bamforth CW & Kanauchi M (2004) Enzymology of vicinal diketone reduction in brewer's yeast. *Journal of the Institute of Brewing* **110**: 83-93.

Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F & Cullin C (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **21**: 3329-3330.

Bergin SA, Allen S, Hession C, Ó Cinnéide E, Ryan A, Byrne KP, Ó Cróinín T, Wolfe KH & Butler G (2022) Identification of European isolates of the lager yeast parent *Saccharomyces eubayanus*. *FEMS Yeast Research* **22**.

Blieck L, Toye G, Dumortier F, Verstrepen KJ, Delvaux FR, Thevelein JM & Dijck PV (2007) Isolation and characterization of brewer's yeast variants with improved fermentation performance under high-gravity conditions. *Applied and Environmental Microbiology* **73**: 815-824.

Blomqvist K, Suihko M-L, Knowles J & Penttilä M (1991) Chromosomal integration and expression of two bacterial α-acetolactate decarboxylase genes in brewer's yeast. *Applied and Environmental Microbiology* **57**: 2796-2803.

Bolat I, Romagnoli G, Zhu F, Pronk JT & Daran JM (2013) Functional analysis and transcriptional regulation of two orthologs of *ARO10*, encoding broad-substrate-specificity 2-oxo-acid decarboxylases, in the brewing yeast *Saccharomyces pastorianus* CBS1483. *FEMS Yeast Research* **13**: 505-517.

Borneman AR, Desany BA, Riches D, Affourtit JP, Forgan AH, Pretorius IS, Egholm M & Chambers PJ (2012) The genome sequence of the wine yeast *VIN7* reveals an allotriploid hybrid genome with *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* origins. *FEMS Yeast Research* **12**: 88-96.

Botman D, de Groot DH, Schmidt P, Goedhart J & Teusink B (2019) *In vivo* characterisation of fluorescent proteins in budding yeast. *Scientific Reports* **9**: 2234.

Brickwedde A, van den Broek M, Geertman JA, Magalhães F, Kuijpers NGA, Gibson B, Pronk JT & Daran JG (2017) Evolutionary engineering in chemostat cultures for improved maltotriose fermentation kinetics in *Saccharomyces pastorianus* lager brewing yeast. *Frontiers in Microbiology* **8**: 1690.

Brouns SJJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJH, Snijders APL, Dickman MJ, Makarova KS, Koonin EV & van der Oost J (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**: 960-964.

Brouwers N, Brickwedde A, Gorter de Vries AR, van den Broek M, Weening SM, van den Eijnden L, Diderich JA, Bai FY, Pronk JT & Daran JG (2019) Himalayan *Saccharomyces eubayanus* genome sequences reveal genetic markers explaining heterotic maltotriose consumption by *Saccharomyces pastorianus* hybrids. *Applied Environmental Microbiology* **85**.

Charpentier E & Doudna JA (2013) Rewriting a genome. *Nature* **495**: 50-51.

Danecek P, Bonfield JK, Liddle J, et al. (2021) Twelve years of SAMtools and BCFtools. *Gigascience* **10**.

Denby CM, Li RA, Vu VT, Costello Z, Lin W, Chan LJG, Williams J, Donaldson B, Bamforth CW, Petzold CJ, Scheller HV, Martin HG & Keasling JD (2018) Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer. *Nature Communications* **9**: 965.

DiCarlo JE, Norville JE, Mali P, Rios X, Aach J & Church GM (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research* **41**: 4336-4343.

Dunn B & Sherlock G (2008) Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. *Genome Research* **18**: 1610-1623.

Duong CT, Strack L, Futschik M, Katou Y, Nakao Y, Fujimura T, Shirahige K, Kodama Y & Nevoigt E (2011) Identification of *Sc*-type *ILV6* as a target to reduce diacetyl formation in lager brewers' yeast. *Metabolic Engineering* **13**: 638-647.

Ekberg J, Rautio J, Mattinen L, Vidgren V, Londesborough J & Gibson BR (2013) Adaptive evolution of the lager brewing yeast *Saccharomyces pastorianus* for improved growth under hyperosmotic conditions and its influence on fermentation performance. *FEMS Yeast Research* **13**: 335-349.

Engler C, Kandzia R & Marillonnet S (2008) A one pot, one step, precision cloning method with high throughput capability. *PLOS ONE* **3**: e3647.

Entian K-D & Kötter P (2007) 25 yeast genetic strain and plasmid collections. *Methods in Microbiology* **36**: 629-666.

Fujii T, Kondo K, Shimizu F, Sone H, Tanaka J & Inoue T (1990) Application of a ribosomal DNA integration vector in the construction of a brewer's yeast having α-acetolactate decarboxylase activity. *Applied and Environmental Microbiology* **56**: 997-1003.

Gangl H, Batusic M, Tscheik G, Tiefenbrunner W, Hack C & Lopandic K (2009) Exceptional

fermentation characteristics of natural hybrids from *Saccharomyces cerevisiae* and *S. kudriavzevii*. *Nature Biotechnology* **25**: 244-251.

Gibson B, Dahabieh M, Krogerus K, Jouhten P, Magalhães F, Pereira R, Siewers V & Vidgren V (2020) Adaptive laboratory evolution of ale and lager yeasts for improved brewing efficiency and beer quality. *Annual Review of Food Science and Technology* **11**: 23-44.

Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA & Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* **6**: 343-345.

Gietz DR & Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods in Enzymology* **350**: 87-96.

Glover NM, Redestig H & Dessimoz C (2016) Homoeologs: what are they and how do we infer them? *Trends in Plant Science* **21**: 609-621.

Godtfredsen SE & Ottesen M (1982) Maturation of beer with α-acetolactate decarboxylase. *Carlsberg Research Communications* **47**: 93-102.

Godtfredsen SE, Rasmussen AM, Ottesen M, Mathiasen T & Ahrenst-Larsen B (1984) Application of the acetolactate decarboxylase from *Lactobacillus casei* for accelerated maturation of beer. *Carlsberg Research Communications* **49**: 69.

Gorter de Vries AR, de Groot PA, van den Broek M & Daran J-MG (2017) CRISPR-Cas9 mediated gene deletions in lager yeast *Saccharomyces pastorianus*. *Microbial Cell Factories* **16**: 222.

Gorter de Vries AR, Voskamp MA, van Aalst ACA, Kristensen LH, Jansen L, van den Broek M, Salazar AN, Brouwers N, Abeel T, Pronk JT & Daran J-MG (2019) Laboratory evolution of a *Saccharomyces cerevisiae* × *S. eubayanus* hybrid under simulated lager-brewing conditions. *Frontiers in Genetics* **10**: 242.

Gorter de Vries AR, Knibbe E, van Roosmalen R, van den Broek M, de la Torre Cortés P, O'Herne SF, Vijverberg PA, el Masoudi A, Brouwers N, Pronk JT & Daran J-MG (2020) Improving industrially relevant phenotypic traits by engineering chromosome copy number in *Saccharomyces pastorianus*. *Frontiers in Genetics* **11**.

Gorter de Vries AR, Pronk JT & Daran J-MG (2019) Lager-brewing yeasts in the era of modern genetics. *FEMS Yeast Research* **19**.

Gorter de Vries AR, Couwenberg LGF, van den Broek M, de la Torre Cortés P, ter Horst J, Pronk JT & Daran J-MG (2018) Allele-specific genome editing using CRISPR–Cas9 is associated with loss of heterozygosity in diploid yeast. *Nucleic Acids Research* **47**: 1362-1372.

Guo W, He X, Tie C & Zhang B (2001) Expression of α-acetolactate decarboxylase gene from *Bacillus subtilis* in brewer's yeast. *Wei Sheng Wu Xue Bao* **41**: 105-108.

Hansen EH, Møller BL, Kock GR, Bünner CM, Kristensen C, Jensen OR, Okkels FT, Olsen CE, Motawia MS & Hansen J (2009) *De novo* biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*). *Applied Environmental Microbiology* **75**: 2765-2774.

Hassing E-J, de Groot PA, Marquenie VR, Pronk JT & Daran J-MG (2019) Connecting central carbon and aromatic amino acid metabolisms to improve *de novo* 2-phenylethanol production in *Saccharomyces cerevisiae*. *Metabolic Engineering* **56**: 165-180.

Hawthorne D & Philippsen P (1994) Genetic and molecular analysis of hybrids in the genus *Saccharomyces* involving *S. cerevisiae*, *S. uvarum* and a new species, *S. douglasii*. *Yeast* **10**: 1285-1296.

Iattici F, Catallo M & Solieri L (2020) Designing new yeasts for craft brewing: When natural biodiversity meets biotechnology. *Beverages* **6**.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA & Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816-821.

Juergens H, Varela JA, Gorter de Vries AR, Perli T, Gast VJM, Gyurchev NY, Rajkumar AS, Mans R, Pronk JT, Morrissey JP & Daran J-MG (2018) Genome editing in *Kluyveromyces* and *Ogataea* yeasts using a broad-host-range Cas9/gRNA co-expression plasmid. *FEMS Yeast Research* **18**.

Knijnenburg TA, Daran J-MG, van den Broek MA, Daran-Lapujade PAS, de Winde JH, Pronk JT, Reinders MJT & Wessels LFA (2009) Combinatorial effects of environmental parameters on transcriptional regulation in *Saccharomyces cerevisiae*: A quantitative analysis of a compendium of chemostat-based transcriptome data. *BMC Genomics* **10**: 53.

Krogerus K, Magalhães F, Vidgren V & Gibson B (2015) New lager yeast strains generated by interspecific hybridization. *Journal of Industrial Microbiology and Biotechnology* **42**: 769-778.

Kuijpers NGA, Solis-Escalante D, Bosman L, van den Broek M, Pronk JT, Daran J-M & Daran-Lapujade P (2013) A versatile, efficient strategy for assembly of multi-fragment expression vectors in *Saccharomyces cerevisiae* using 60 bp synthetic recombination sequences. *Microbial Cell Factories* **12**: 47-47.

Langdon QK, Peris D, Baker EP, Opulente DA, Nguyen HV, Bond U, Gonçalves P, Sampaio JP, Libkind D & Hittinger CT (2019) Fermentation innovation through complex hybridization of wild and domesticated yeasts. *Nature Ecology & Evolution* **3**: 1576-1586.

Le Jeune C, Lollier M, Demuyter C, Erny C, Legras J-L, Aigle M & Masneuf-Pomarède I (2007) Characterization of natural hybrids of *Saccharomyces cerevisiae* and *Saccharomyces*

bayanus var. uvarum. *FEMS Yeast Research* **7**: 540-549.

Lee ME, DeLoache WC, Cervantes B & Dueber JE (2015) A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synthetic Biology* **4**: 975-986.

Li H (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv: *Genomics*.

Libkind D, Hittinger CT, Valério E, Gonçalves C, Dover J, Johnston M, Gonçalves P & Sampaio JP (2011) Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences* **108**: 14539-14544.

Lombardi L, Bergin SA, Ryan A, Zuniga-Soto E & Butler G (2022) CRISPR-Cas9 editing induces loss of heterozygosity in the pathogenic yeast *Candida parapsilosis*. *mSphere* e0039322.

Lõoke M, Kristjuhan K & Kristjuhan A (2011) Extraction of genomic DNA from yeasts for PCR-based applications. *BioTechniques* **50**: 325-328.

Louis VL, Despons L, Friedrich A, et al. (2012) *Pichia sorbitophila*, an interspecies yeast hybrid, reveals early steps of genome resolution after polyploidization. *G3 Genes|Genomes|Genetics* **2**: 299-311.

Ma H, Marti-Gutierrez N, Park SW, *et al.* (2017) Correction of a pathogenic gene mutation in human embryos. *Nature* **548**: 413-419.

Marçais G, Delcher AL, Phillippy AM, Coston R, Salzberg SL & Zimin A (2018) MUMmer4: A fast and versatile genome alignment system. *PLOS Computational Biology* **14**: e1005944.

Masneuf I, Hansen J, Groth C, Piskur J & Dubourdieu D (1998) New hybrids between *Saccharomyces sensu stricto* yeast species found among wine and cider production strains. *Applied Environmental Microbiology* **64**: 3887-3892.

Nakao Y, Kanamori T, Itoh T, Kodama Y, Rainieri S, Nakamura N, Shimonaga T, Hattori M & Ashikari T (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Research* **16**: 115-129.

Nijkamp JF, van den Broek MA, Geertman J-MA, Reinders MJT, Daran J-MG & de Ridder D (2012) *De novo* detection of copy number variation by co-assembly. *Bioinformatics* **28**: 3195-3202.

Okuno M, Kajitani R, Ryusui R, Morimoto H, Kodama Y & Itoh T (2016) Next-generation sequencing analysis of lager brewing yeast strains reveals the evolutionary history of interspecies hybridization. *DNA Research* **23**: 67-80.

Ortiz-Merino RA, Kuanyshev N, Braun-Galleani S, Byrne KP, Porro D, Branduardi P & Wolfe KH (2017) Evolutionary restoration of fertility in an interspecies hybrid yeast, by whole-genome duplication after a failed mating-type switch. *PLOS Biology* **15**: e2002128.

Quinlan AR & Hall IM (2010) BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**: 841-842.

Salazar AN, Gorter de Vries AR, van den Broek M, Brouwers N, de la Torre Cortès P, Kuijpers NGA, Daran J-MG & Abeel T (2019) Chromosome level assembly and comparative genome analysis confirm lager-brewing yeasts originated from a single hybridization. *BMC Genomics* **20**: 916.

Sone H, Fujii T, Kondo K, Shimizu F, Tanaka J & Inoue T (1988) Nucleotide sequence and expression of the Enterobacter aerogenes α-acetolactate decarboxylase gene in brewer's yeast. *Applied and Environmental Microbiology* **54**: 38-42.

Steensels J, Snoek T, Meersman E, Nicolino MP, Voordeckers K & Verstrepen KJ (2014) Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiology Reviews* 38: 947-995.

Suihko ML, Blomqvist K, Penttilä M, Gisler R & Knowles J (1990) Recombinant brewer's yeast strains suitable for accelerated brewing. *Journal of Biotechnology* **14**: 285-300.

Turgeon Z, Sierocinski T, Brimacombe CA, Jin Y, Goldhawke B, Swanson JM, Husnik JI & Dahabieh MS (2021) Industrially applicable *de novo* lager yeast hybrids with a unique genomic architecture: creation and characterization. *Applied Environmental Microbiology* **87**.

van den Broek M, Bolat I, Nijkamp JF, Ramos E, Luttik MA, Koopman F, Geertman JM, de Ridder D, Pronk JT & Daran JM (2015) Chromosomal copy number variation in *Saccharomyces pastorianus* is evidence for extensive genome dynamics in industrial lager brewing strains. *Applied Environmental Microbiology* **81**: 6253-6267.

Wach A, Brachat A, Pöhlmann R & Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793-1808.

Walther A, Hesselbart A & Wendland J (2014) Genome sequence of *Saccharomyces carlsbergensis*, the world's first pure culture lager yeast. *G3 Genes|Genomes|Genetics* **4**: 783-793.

Yamano S, Tanaka J & Inoue T (1994) Cloning and expression of the gene encoding α-acetolactate decarboxylase from *Acetobacter aceti ssp. xylinum* in brewer's yeast. *Journal of Biotechnology* **32**: 165-171.

Yamano S, Tomizuka K, Sone H, Imura M, Takeuchi t, Tanaka J & Inoue T (1995) Brewing performance of a brewer's yeast having α-acetolactate decarboxylase from *Acetobacter aceti subsp. xylinum*. *Journal of Biotechnology* **39**: 21-26.

Chapter 5

Unlocking lager's flavour palette by metabolic engineering of *Saccharomyces pastorianus* for enhanced ethyl ester production

Nicole X. Bennis, Jimme Bieseman & Jean-Marc G. Daran

This chapter has been published in Metabolic Engineering (2024)

Abstract

Despite being present in trace amounts, ethyl esters play a crucial role as flavour compounds in lager beer. In yeast, ethyl hexanoate, ethyl octanoate and ethyl decanoate, responsible for fruity and floral taste tones, are synthesized from the toxic medium chain acyl-CoA intermediates released by the fatty acid synthase complex during the fatty acid biosynthesis, as a protective mechanism. The aim of this study was to enhance the production of ethyl esters in the hybrid lager brewing yeast *Saccharomyces pastorianus* by improving the medium chain acyl-CoA precursor supply. Through CRISPR-Cas9-based genetic engineering, specific *FAS1* and *FAS2* genes habouring mutations in domains of the fatty acid synthase complex were overexpressed in a single and combinatorial approach. These mutations in the *ScFAS* genes led to specific overproduction of the respective ethyl esters: overexpression of $ScFAS1^{I306A}$ and $ScFAS2^{G1250S}$ significantly improved ethyl hexanoate production and $ScFAS1^{R1834K}$ boosted the ethyl octanoate production. Combinations of *ScFAS1* mutant genes with $ScFAS2^{G1250S}$ greatly enhanced predictably the final ethyl ester concentrations in cultures grown on full malt wort, but also resulted in increased levels of free medium chain fatty acids causing alterations in flavour profiles. Finally, the elevated medium chain fatty acid pool was directed towards the ethyl esters by overexpressing the esterase *ScEEB1*. The genetically modified *S. pastorianus* strains were utilized in lager beer production, and the resulting beverage exhibited significantly altered flavour profiles, thereby greatly expanding the possibilities of the flavour palette of lager beers.

5.1. Introduction

The spectrum of flavour molecules that defines the taste of beer is highly diverse. The interplay of these flavour molecules at varying concentrations significantly broadens the potential flavour profiles achievable in a beverage. Additionally, synergism, where flavour molecules can intensify or dampen specific aromas, even below the human sensory threshold (HST), is noteworthy (Verstrepen *et al.*, 2003a, Pires *et al.*, 2014). Traditionally, hops play a dominant role as the primary flavour-contributing ingredient in lager brewing, but the role of brewing yeast, *Saccharomyces pastorianus*, is central for flavour enhancement during fermentation. This yeast (Dunn & Sherlock, 2008, Nakao *et al.*, 2009, Salazar *et al.*, 2019), an interspecific hybrid of *Saccharomyces cerevisiae* and the cryotolerant *Saccharomyces eubayanus* (Libkind *et al.*, 2011), naturally produces a wide spectrum of molecules with aromatic properties, including higher alcohols, carbonyl compounds, vicinal diketones, and esters (Nordström, 1964, Peddie, 1990, Renger *et al.*, 1992, Verstrepen *et al.*, 2003a, Pires *et al.*, 2014).

Esters, present in only trace amounts and still being the most important aromas (Pires *et al.*, 2014), can be categorized into two groups based on their precursors: acetate esters (e.g., isoamyl acetate, ethyl acetate and phenylethyl acetate) and ethyl esters (e.g., ethyl hexanoate, ethyl octanoate) (Figure 5.1A). The formation of acetate esters involves fusel alcohols, products of the Ehrlich pathways that is involved in degradation of carbon skeleton of some class of amino acids, or ethanol and acetyl-CoA (Hazelwood *et al.*, 2008, Romagnoli *et al.*, 2012). This acetylation reaction is performed by alcohol acetyltransferases (AATases) encoded by *ATF1* and *ATF2* (Fujii *et al.*, 1994, Fujii *et al.*, 1996, Yoshimoto *et al.*, 1998, Yoshimoto *et al.*, 1999, Mason & Dufour, 2000, Verstrepen *et al.*, 2003b). On the other hand, ethyl esters, the class discussed in this manuscript, are formed by the condensation of ethanol and an acyl-CoA of which the carbon chain may vary from two to ten. Acyl-CoA and ethanol are converted to their ethyl ester derivative by acyl-coenzymeA:ethanol O-acyltransferase esterases (AEAT) encoded by *EEB1* (Saerens *et al.*, 2006) and *EHT1* (Mason & Dufour, 2000, Saerens *et al.*, 2006, Knight *et al.*, 2014), with *EEB1* encoding the main enzyme responsible for this conversion (Saerens *et al.*, 2006). Several hypotheses have been postulated regarding the physiological role of ester biosynthesis, but the most pertinent remains that ester formation contributes to detoxification of higher alcohols and medium chain fatty acids (MCFA) (Saerens *et al.*, 2010). In the case of ethyl esters, their toxicity is lower than that of their respective acids, especially butanoic (C_4), hexanoic (C_6) and octanoic acid (C_8) (Hundová & Fencl, 1977, Legras *et al.*, 2010, Besada-Lombana *et al.*, 2017, Baumann *et al.*, 2021). Therefore, the formation of ethyl esters mitigate accumulation of the acid and facilitate export by diffusion across the plasma membrane which as well helps to shift the reaction equilibrium towards the ester biosynthesis (Nordström, 1964, Saerens *et al.*, 2010, Liu *et al.*, 2013).

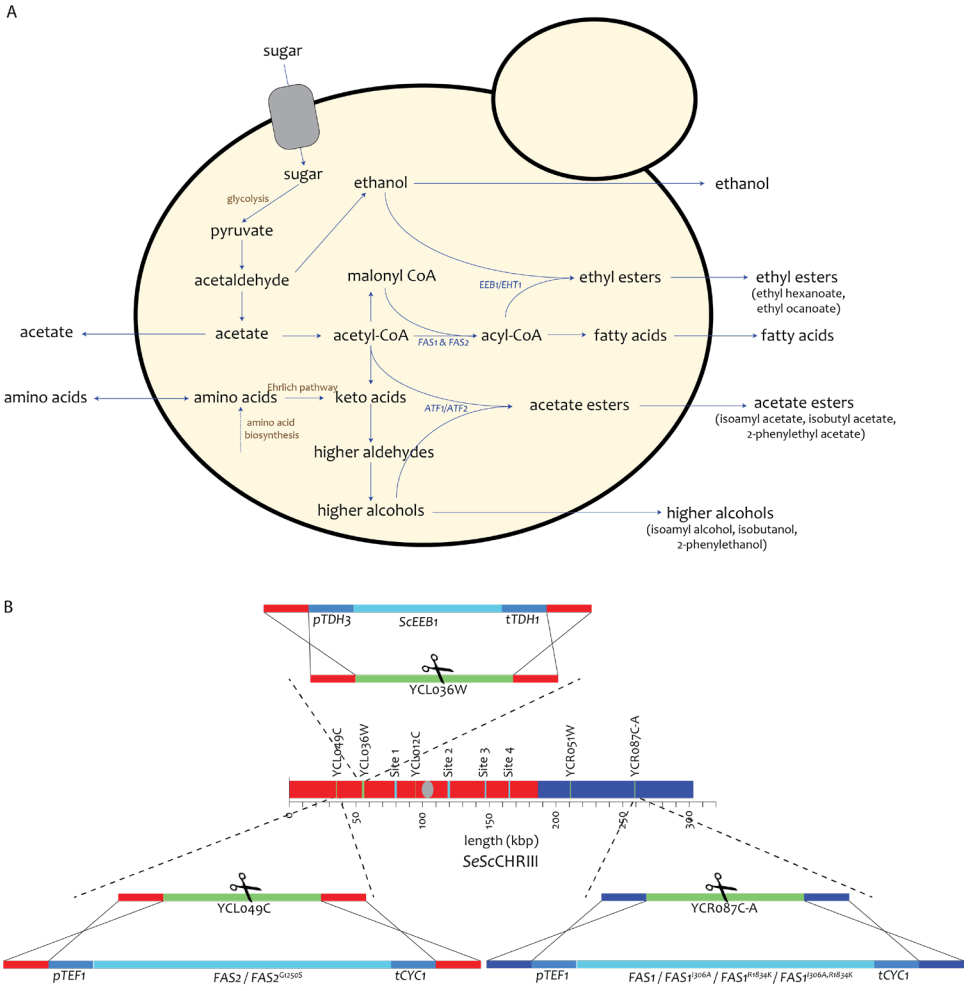


Figure 5.1. A) Overview of metabolic pathways involved in ester biosynthesis in brewing yeasts. **B)** Strategy for integration of *ScFAS1* and *ScFAS2* genes and mutant variants in *S. pastorianus* CBS 1483 based on CRISPR-Cas9. Integration sites *Se*YCL049 (*ScFAS2* and *ScFAS2*^{G1250S}) and *Sc*YCR087C-A (*ScFAS1*, *ScFAS1*^{I306A}, *ScFAS1*^{R1834K} and *ScFAS1*^{I306A,R1834K}) on the hybrid *Se*ScCHRIII (4 copies) were chosen. The *ScEEB1* gene was overexpressed in the genomic location *Se*YCL036W on the *Se*ScCHRIII (4 copies).

In brewing context, attempts to enhance ethyl ester concentration above human sensory threshold by overexpressing acyl-coenzymeA:ethanol O-acyltransferase esterases *EEB1* and *EHT1* remained largely unsuccessful (Table 5.1) (Saerens *et al.*, 2006, Yin *et al.*, 2019). This absence of phenotype suggests that native acyl-coenzymeA:ethanol O-acyltransferase esterase capacity may not be limiting, but rather the precursor concentrations (Saerens *et al.*, 2006, Saerens *et al.*, 2008). Hitherto, no studies attempted to deregulate the supply of short and medium acyl CoA in *Saccharomyces* species alone or in combination with the overexpression of an acyl-coenzymeA:ethanol O-acyltransferase esterases. Thus, improving acyl-CoA precursor

supply sounds a logical metabolic engineering target to enhance and tune ethyl ester formation.

In *Saccharomyces cerevisiae*, the fatty acid synthase (FAS) complex consists of two subunits, the β-chain encoded by *FAS1* that harbours the domains acetyl transferase (AT), enoyl reductase (ER), dehydratase (DH) and malonyl/palmitoyl transferase (MPT) and the α-chain encoded by *FAS2* that harbours the domains acyl carrier protein (ACP), ketoacyl reductase (KR), ketoacyl synthase (KS) and the phosphopantetheine transferase (PPT). The fatty acid synthesis starts as a two-carbon chain from acetyl-CoA, that is extended two carbons at a time by the addition of an acetyl group from malonyl-CoA. The KS domain is responsible for the condensation of an acyl substrate (either the starter molecule acetyl or an ACP-bound elongated acyl-chain) with malonyl moieties. The resulting β-ketoacyl intermediate is processed sequentially by the KR, DH and ER domains, to a fully reduced acyl-chain. The resulting acyl-chain is processed in a subsequent cycle. The process repeats till the final fatty acid is cleaved off, which in *S. cerevisiae* happens typically when the fatty acid reaches 16 or 18 carbons (Wakil *et al.*, 1983). Thus, MCFA with carbon length ranged from C6 to C10, are only intermediates of this biosynthetic process probably originating from premature release of the nascent fatty acids.

Several approaches, including overexpression of *ScFAS1* and *ScFAS2* genes (Furukawa *et al.*, 2003) or expression of thioesterases with specificities for shorter acyl-CoA successfully resulted in the production of MCFA (Leber *et al.*, 2015). Moreover, specific mutations in *FAS1* or *FAS2* genes, particularly in active sites involved in chain elongation, yielding rate imbalance between the coordinated steps in fatty acid biosynthesis, have been demonstrated to enhance and tune MCFA release in *S. cerevisiae* for various purposes (e.g. beverages (Aritomi *et al.*, 2004) and fine chemical production (Fernandez-Moya & Da Silva, 2017, Gajewski *et al.*, 2017a, Gajewski *et al.*, 2017b)). The I306A mutation in the AT domain of *FAS1* causes an elevated acetyl uptake. By forcing the initiation of a new fatty acid production cycle instead of elongating the existing chain, the current elongated chain is released from the FAS complex in shorter fatty acid form (Gajewski *et al.*, 2017b). Additionally, the mutation R1834K affects the MPT domain of *FAS1* by making it less favourable for malonyl to bind. As malonyl is used for elongating the existing fatty acid chain, this mutation will result in early termination of the iterating elongation cycle (Gajewski *et al.*, 2017b). Lastly, the G1250S mutation in the KS domain in *FAS2* prevents short chain intermediates from entering the KS domain and thus being released (Aritomi *et al.*, 2004, Gajewski *et al.*, 2017b).

In the present study, we aimed to modify the ethyl ester profile in *S. pastorianus* by augmenting the supply of the precursors MCFAs. This was achieved by introducing mutations in specific domains of the fatty acid synthesis complex and overexpressing single and combinatorial mutants of *ScFAS1* and *ScFAS2*, and overexpressing the

Table 5.1. Flavour compounds found in beer, with their corresponding Human Sensory Threshold (HST) value obtained from literature, and their concentrations in lager beer brewed with CBS 1483 in tall European Brewing Convetion (E.B.C.) tubes on 17 P° wort as a reference (Brickwedde *et al.*, 2017), [a] (Stewart, 2017), [b] (Olaniran *et al.*, 2017), [c] (Engan, 1972), [d] (Ferreira & Guido, 2018), [e] (Meilgaard, 1982), [f] (Xi *et al.*, 2011), [g] (Schieberle & Hofmann, 1997).

Flavour Compound	Taste	HST (mg L^{-1})	Concentrations in lager beer (mg L^{-1})
Acetate esters			
Ethyl acetate	Sweet, fruity, solvent-like	3.0[a], 20-40[e]	49.38
Isobutyl acetate	Fruity, banana, pineapple	0.7[a]	0.16
Isoamyl acetate	Banana, pear	1.2[a]	5.26
Phenylethyl acetate	Rose, honey	0.4[a]	-
Ethyl esters			
Ethyl butanoate	Sweet, fruity, berry	0.001[g]	0.68
Ethyl hexanoate	Apple, fruity, sweet	0.15-0.25[e], 0.22[a]	0.43
Ethyl octanoate	Apple, aniseed	0.9[a]	0.76
Ethyl decanoate	Floral	0.2[f]	0.16
Higher alcohols			
Isoamyl alcohol	Alcohol	65[a]	68.31
Isobutyl alcohol	Solvent	70[a]	13.40
Propanol	Solvent	200[a]	-
2-Methyl butanol	Banana, medicinal	65[a]	-
Phenylethyl alcohol	Roses, sweet	125[a]	-
Carbonyl compounds			
Acetaldehyde	Grass, green apple	10-20[b]	-
Vicinal diketones			
Diacetyl	Buttery, butterscotch	0.07-0.15[e], 0.15[d]	82.13*
2,3-pentanedione	Fermented dairy	0.015[d]	25.81

* Lagering period was unfinished in this fermentation

esterase *EEB1*. This genetic engineering approach resulted in elevated levels of specific ethyl esters and alterations in flavour profiles. The genetically modified *S. pastorianus* strains were subsequently employed for lager beer production, and the resulting beverages underwent sensory evaluation to assess the modified flavour profile.

5.2. Materials and Methods

Strains and cultivation conditions

All yeast strains used in this study are listed in Table 5.2. Yeast were grown on complex medium that contained 10.0 g L^{-1} bacto yeast extract, 20.0 g L^{-1} bacto peptone and 20 g L^{-1} glucose (YPD). For selection of transformants or when selective pressure was required, YPD medium was supplemented with 200 mg L^{-1} of hygromycin (YPD hygR). Solid YPD media was obtained by addition of 20 g L^{-1} bacto agar. *S. cerevisiae* and *S. pastorianus* strains were cultivated at 30 °C and 20 °C, respectively, while shaking at 200 rpm in an Innova 43/43R incubation shaker (Brunswick, Nijmegen, the Netherlands). On solid media, *S. pastorianus* strains were incubated at 20 °C in a temperature controlled room. Fermentations were performed with full malt wort at either 17 °P or 5.7 °P (Heineken, Zoeterwoude, The Netherlands) supplemented with 1 mL L^{-1} pluronic acid 6100 PE antifoam (BASF, Ludwigshafen, Germany).

Escherichia coli XL1-Blue (New England Biolabs, Ipswich, MA) cultures were grown in 15 mL Greiner tubes containing 5 mL lysogenic broth (LB) medium supplemented with 10 mg L^{-1} ampicillin (LB Amp) for selection and cultivated at 37 °C while shaking at 200 rpm in an Innova 4000 Incubator Shaker (Eppendorf AG, Hamburg, Germany). Solid medium was prepared by addition of 20 g L^{-1} bacto agar to the medium.

Frozen stock cultures of *S. pastorianus*, *S. cerevisiae* and *E. coli* strains were prepared by addition of 30% (v/v) glycerol and stocks were stored as 1 mL aliquots at -80 °C.

Molecular biology techniques

PCR amplifications for cloning purposes were performed using Phusion High Fidelity Polymerase (Thermo Fisher Scientific, Landsmeer, The Netherlands) according to the manufacturer's instructions. Diagnostic PCRs were performed with DreamTaq PCR Mastermix (Thermo Fisher Scientific) according to the manufacturer's instructions. All plasmids and primers (Sigma Aldrich, Zwijndrecht, The Netherlands) constructed or used in this study are listed in Table 5.3 and Table 5.4, respectively. DNA was visualized by electrophoresis on 1% agarose gels with the GeneRuler™ Ladder Mix (Thermo Scientific) for size verifications. PCR products were purified from gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Alternatively, PCR mixtures were digested with FastDigest DpnI enzyme (Thermo Fisher Scientific) to remove circular templates. Digested PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) following the manufacturer's instructions using milliQ as eluent solvent. Gibson Assembly was performed with 200 fmol of each fragment using NEBuilder® HiFi DNA Assembly Master Mix (New England BioLabs) and one hour incubation at 50 °C (Gibson *et al.*, 2009). Gate assembly was done according to Lee *et al.*, 2015 with 20 fmol of each

Table 5.2. Strains used in this study

Strain	Relevant genotype	Reference
CEN.PK113-7D	*MATa MAL2-8c SUC2*	(Entian & Kötter, 2007)
CBS 1483	Group II brewer's yeast, Heineken bottom yeast, isolated July 1927	(Dunn & Sherlock, 2008)
IMI541	CBS 1483 Δ*ScYCR087C::ScFAS1*	This study
IMI542	CBS 1483 Δ*ScYCR087C::ScFAS1*^{I306A}	This study
IMI543	CBS 1483 Δ*ScYCR087C::ScFAS1*^{R1834K}	This study
IMI544	CBS 1483 Δ*ScYCR087C::ScFAS1*^{I306A,R1834K}	This study
IMI545	CBS 1483 Δ*SeYCL049C::ScFAS2*	This study
IMI546	CBS 1483 Δ*SeYCL049C::ScFAS2*^{G1250S}	This study
IMI547	CBS 1483 Δ*SeYCL049C::ScFAS2* Δ*ScYCR087C::ScFAS1*	This study
IMI548	CBS 1483 Δ*SeYCL049C::ScFAS2* Δ*ScYCR087C::ScFAS1*^{I306A}	This study
IMI549	CBS 1483 Δ*SeYCL049C::ScFAS2* Δ*ScYCR087C::ScFAS1*^{R1834K}	This study
IMI550	CBS 1483 Δ*SeYCL049C::ScFAS2* Δ*ScYCR087C::ScFAS1*^{I306A,R1834K}	This study
IMI551	CBS 1483 Δ*SeYCL049C::ScFAS2*^{G1250S} Δ*ScYCR087C::ScFAS1*	This study
IMI552	CBS 1483 Δ*SeYCL049C::ScFAS2*^{G1250S} Δ*ScYCR087C::ScFAS1*^{I306A}	This study
IMI553	CBS 1483 Δ*SeYCL049C::ScFAS2*^{G1250S} Δ*ScYCR087C::ScFAS1*^{R1834K}	This study
IMI554	CBS 1483 Δ*SeYCL049C::ScFAS2*^{G1250S} Δ*ScYCR087C::ScFAS1*^{I306A,R1834K}	This study
IMI577	CBS 1483 Δ*SeYCL049C::ScFAS2*^{G1250S} Δ*ScYCR087C::ScFAS1*^{I306A,R1834K} Δ*SeYCL036W::ScEEB1*	This study
IMI578	CBS 1483 Δ*SeYCL036W::ScEEB1*	This study

fragment with restriction enzyme BsaI (Thermo Fisher Scientific) and T7 DNA ligase (Thermo Fisher Scientific). Constructed plasmids were transformed to *E. coli* XL1-blue (New England Biolabs) chemically competent cells. Plasmids were isolated from the *E. coli* cells with the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the supplier's manual with milliQ as eluent solvent. Diagnostic plasmid digestions were performed using Fast Digest enzymes (Thermo Fisher Scientific) and the Green Fast Digest Buffer (Thermo Fisher Scientific) on 500 ng plasmid DNA.

Plasmid construction

The plasmids expressing *S. cerevisiae FAS* genes were constructed via Gibson assembly (Gibson *et al.*, 2009). The plasmid backbone (p426-*pTEF1-amdS*) containing the *ScTEF1* promotor, *ScCYC1* terminator, *E. coli pBR322* origin of replication and ampicillin resistance marker and the yeast 2µm origin of replication and *Aspergillus niger amdS* auxotrophic marker (Solis-Escalante *et al.*, 2013) was linearized by PCR amplification with primers 5921 & 7812. To introduce mutations in the *ScFAS1* and *ScFAS2* genes, the genes were PCR amplified from the *S. cerevisiae* CEN.PK113-7D genome using primers containing the mutations, and split into two or three parts depending on the number of mutations, with each fragment containing homology flanks for proper assembly. For *ScFAS1*, two fragments were obtained with primers 19699 & 19710 and 19748 & 19700 and assembled into the p426-*pTEF1-amdS* backbone, resulting in pUD1295; *ScFAS1*^{I306A} fragments with primer pairs 19699 & 19703 and 19704 & 19700 were assembled resulting in pUD1296; *ScFAS1*^{R1834K} fragments with primer pairs 19699 & 19705 and 19706 &19700 were assembled and resulting in pUD1297; and for *ScFAS1*^{I306A,R1834K} three fragments amplified with primers 19699 & 19703, 19704 & 19705 and 19706 & 19700 were assembled resulting in pUD1298. For *ScFAS2*, two fragments were obtained with primers 19701 & 19742 and 19743 & 19702, assembled into the p426-*pTEF1-amdS* backbone and resulted in pUD1299; *ScFAS2*^{G1250S} fragments were obtained with primer pairs 19701 & 19707 and 19708 & 19702 and assembled resulting in pUD1300.

To verify the correct assembly of the *FAS* genes into the backbone, diagnostic PCRs were performed with primers 2399 & 2626. Additionally, restriction analysis of the plasmids was performed with for the *ScFAS1* encoding plasmid the restriction enzymes BamHI and KpnI and for the *ScFAS2* encoding plasmids the restriction enzymes PvuI and EcoRI.

The primers used to sequence the *ScFAS1* genes on the plasmids and PCR-amplified integration regions were 19699, 19700, 19709, 19710, 19748, 19749, 19750, 19751, 19752, 19753, 19754 and 19706 (only *ScFAS1*^{R1834K}). The primers used to sequence the *ScFAS2* genes on the plasmids and PCR-amplified integration regions were 19701, 19702, 19711, 19712, 19755, 19756, 19757, 19758, 19759, 19760, 19761, 19762, 19707 (only *ScFAS2*^{G1250S}), 19708 (only *ScFAS2*^{G1250S}), 19742 (only *ScFAS2*) and 19743 (only *ScFAS2*).

The *ScEEB1* gene was PCR amplified from *S. cerevisiae* CEN.PK113-7D genomic DNA using primers 19949 & 19950. The PCR product was cloned into the pYTK001 entry vector for golden gate cloning according to (Lee *et al.*, 2015). Golden gate with pYTK009 (*pTDH3*), pGGKp395 (*ScEEB1*), pYTK056 (*tTDH1*) (Lee *et al.*, 2015) and backbone pGGKd015 (Hassing *et al.*, 2019), resulting in plasmid pUD1325, which was verified using diagnostic primers 10320 & 10325. pUD1325 was sequence verified by whole plasmid sequence (Plasmidsaurus, Eugene, OR, USA).

Table 5.3. Plasmids used in this study.

Plasmid	Genotype	Reference
pUDP269	*ori bla* panARS^{OPT} *AgpTEF1-hph-AgtTEF1 ScpTDH3-HH-gRNA SeYCL049C-HDV-SctCYC1 AapTEF1-Spcas9$^{D147Y\ P411T}$-SctPHO5*	(Bennis *et al.*, 2023)
pUDP270	*ori bla* panARS^{OPT} *AgpTEF1-hph-AgtTEF1 ScpTDH3-HH-gRNA SeYCL036W-HDV-SctCYC1 AapTEF1-Spcas9$^{D147Y\ P411T}$-SctPHO5*	(Bennis *et al.*, 2023)
pUDP273	*ori bla* panARS^{OPT} *AgpTEF1-hph-AgtTEF1 ScpTDH3-HH-gRNA ScYCR087C-A-HDV-SctCYC1 AapTEF1-Spcas9$^{D147Y\ P411T}$-SctPHO5*	(Bennis *et al.*, 2023)
p426-TEF1-amds	*ori bla* 2µm *pTEF1-amdSYM-tTEF1 pTEF1*-empty-*tCYC1*	(Solis-Escalante *et al.*, 2013)
pUD1295	*ori bla* 2µm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1-tCYC1*	This study
pUD1296	*ori bla* 2µm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1^{I306A}-tCYC1*	This study
pUD1297	*ori bla* 2µm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1^{R1834K}-tCYC1*	This study
pUD1298	*ori bla* 2µm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1^{I306A,R1834K}-tCYC1*	This study
pUD1299	*ori bla* 2µm *pTEF1-amdS-tTEF1 pTEF1-ScFAS2-tCYC1*	This study
pUD1300	*ori bla* 2µm *pTEF1-amdS-tTEF1 pTEF1-ScFAS2^{G1250S}-tCYC1*	This study
pGGKd015	*ampR-ColE1* GFP dropout	(Hassing *et al.*, 2019)
pYTK009	*camR pTDH3*	(Lee *et al.*, 2015)
pYTK056	*camR TDH1t*	(Lee *et al.*, 2015)
pGGKp395	*camR ScEEB1*	This study
pUD1325	*ori bla pTDH3-ScEEB1-tTDH1*	This study

Construction of yeast strains

CRISPR-Cas9 genome editing in *S. pastorianus* strains was performed by transforming *S. pastorianus* strains with 500 ng gRNA plasmid and 1000 ng repair fragment. As negative control, transformations from which the repair fragment was omitted, were performed. Yeast transformation was performed by electroporation using 50 µL of competent cells in 0.2 cm chilled Gene Pulser® Cuvettes (Bio-Rad) and pulsed with for 5 ms with 1.5 kV by the MicroPulser (Serial No. 411BR5600, Bio-Rad). The transformed cells were incubated in 0.5 mL YPD during 2 h, followed by re-suspending in 100 µL sterile demi-water and plating on selective medium (Gorter de Vries *et al.*, 2017).

The *ScFAS* gene integrations were performed by co-transforming the gRNA plasmid and the corresponding repair fragment consisting of the *ScFAS* expression cassette flanked by 60 bp homologous sequences for recombination. The *ScFAS1*, *ScFAS1^{I306A}*, *ScFAS1^{R1834K}* and *ScFAS1^{I306A,R1834K}* repair fragments were obtained by PCR amplification using primer pair 19744 & 19745, which incorporate compatible flanks for the ScYCR087C integration site (Bennis *et al.*, 2023), and pUD1295, pUD1296, pUD1297 and

Table 5.4. Primers used in this study.

Number	Sequence (5' -> 3')	Purpose
FAS expressing plasmid construction		
2399	AACACCCAAGCACAGCATAC	Diagnostic verification of *FAS* genes into p426-*pTEF1-amdS*
2626	GGTTGAAGAAGTCGCTATC	
5921	CGAGTAATCTTTCTTTCGTATCGTTAGATTAGAT-TCAAAA	p426-*pTEF1-amdS* backbone amplification
7812	TCATGTAATTAGTTATGTCACGCTTACATTC	
19699	GCTCATTAGAAAGAAAGCATAGCAATCTAATCTA-AGTTTTATGGACGCTTACTCCACAAG	Amplification *FAS1* with p426-*pTEF1-amdS* backbone compatible flanks
19700	GGAGGGCGTGAATGTAAGCGTGACATAACTAATTA-CATGATTAGGATTGTTCATACTTTTCCCAGTTG	
19701	GCTCATTAGAAAGAAAGCATAGCAATCTAATCTA-AGTTTTATGAAGCCGGAAGTTGAGCAAG	Amplification *FAS2* with p426-*pTEF1-amdS* backbone compatible flanks
19702	GGAGGGCGTGAATGTAAGCGTGACATAACTAATTA-CATGACTATTTCTTAGTAGAAACGGCGACC	
19703	GCAATTACTGTATTATTCTTCGCTGGTGTTCGTTGTTAC-GAAGCATACCCAAACACTTCC	Introduction I306A mutation in *FAS1*
19704	CGTAACAACGAACACCAGCGAAGAATAATACAGTA-ATTGCTTTTCTTACGGAGACG	
19705	TAGTTGAAGTTGTGTTCTACAAAGGTATGACTATG-CAAGTTGCTGTTCC	Introduction R1834K mutation in *FAS1*
19706	ACTTGCATAGTCATACCTTTGTAGAACA-CAACTTCAACTAAAGATTCGATAGAC	
19707	TGAGGTTGGTAACTGTTCTGGTTCTTCTATGGGTGGT-GTTTCTGCCTTACG	Introduction G1250S mutation in *FAS2*
19708	AACACCACCCATAGAAGAACCAGAACAGTTAC-CAACCTCAGAAACATGTACG	
19710	TCAACTTGTTTACCAGCTGGC	Amplification of *FAS1* in two fragments
19748	GTAAATAAGACTAACTCTCATTTGCC	Amplification of *FAS1* in two fragments
19742	CCACCCATACCAGAACCAGAACAGTTACCAACCT-CAGAAACATGTACG	Amplification of *FAS2* in two fragments
19743	GGTTGGTAACTGTTCTGGTTCTGGTATGGGTGGT-GTTTCTGCCTTACG	Amplification of *FAS2* in two fragments

Table 5.4. (continued)

Number	Sequence (5' -> 3')	Purpose
Sequencing of *FAS1* and *FAS2*		
19709	GCCGATGCTACTTTTGCTGG	Sequencing *FAS1* genes
19749	CTTCCAATGATGGGACACCAGC	
19750	CTTGACCATTGGTGCTGGTG	
19751	GCCTTTTGTTCCAGTTTTGG	
19752	CTCGAATCTACGATCCAAAACTGG	
19753	CTTCAATAAGTCACCATCGACCG	
19754	GATGGTGACTTATTGAAGTTGG	
19711	GGCTTTGAGATTTGACCGTTTGG	Sequencing *FAS2* genes
19712	GCTGGAGTTGGAGCAGGAG	
19755	CTAAAAATGGACTTGGATAACGG	
19756	GCTTGAAGTTCAGCAACAGTGTCC	
19757	CGTCTTGATCACTGGTGCTG	
19758	GCACCAATAGAACCCTTACCAGC	
19759	GGCAATAGCGCTGATGCTGC	
19760	GGGAAGTCCAGTTGAATGTTAGC	
19761	GGCTAATGACAAGAACGAATCTGCC	
19762	GGGATTACCTTCAGATCTACCC	
ScEEB1 cloning		
19949	AAAGCATCGTCTCATCGGTCTCATATGTTTCGCTCGG-GTTACTATC	Amplification *ScEEB1* from CEN.PK113-7D genomic DNA and cloning as YTK type 3 part
19950	TTTATGCCGTCTCAGGTCTCAGGATTTATAAAACTAACT-CATCAAAGCTGCC	
10320	CATGCGCGGATGACACGAAC	Diagnostic primers for pUD1325 verification
10325	AGTCATCCGAGCGTGTATTG	
Yeast genomic integration		
19744	AAAAGATGAAACCGAGTAAGCTGCTACATAATGTC-TATATATCTACACATAAAATTCCGACATAGCTTCAAAAT-GTTTCTACTCCTTTTTTACTCTTCC	Adds complimentary flanks for ScYCR087C site
19745	TAAGAGTATTCTGTATACAACAGCAAACGGTCTCAGT-CAAGAAATATTTGTTATTACAGGGGCCGCAAATTA-AAGCCTTCGAG	
18534	GAATACCTCTTCGAAACGTTGAG	Diagnostic PCR for ScYCR087C integrations
18535	ATGAGTGGACTGGCAGC	

Table 5.4. (continued)

Number	Sequence (5' -> 3')	Purpose
19763	TTCTATAGATGTACGTAAAGTTCT-GCTCTTCTTTTATAGATAAGAGGATT-GAGACTCGCCCATAGCTTCAAAATGTTTC-TACTCCTTTTTTACTCTTCC	Adds complimentary flanks for *Se*YCL049C site
19764	AGCTCAAGAAAAACCAGGCTATTCGCAGAACAGGA-TAACCAGCTCTGTAACCACATCAATGGCCGCAAATTA-AAGCCTTCGAG	
18514	TCAGTTAGAGTGACAGTTGC	Diagnostic PCR for *Sc*YCR087C integrations
18515	GTTGTGCGTTTTACGTGC	
18517	AGATGCGGCAGCCAGTGAGGGCGTTGGGCATGATC-GAAAGCCAAGACCCACCAATTCGAGACTGGCCGATA-ATTGCAGACG	Adds complimentary flanks for *Se*YCL036W site
18518	ATATACATATACGTACGTATGTATCTA-CAGAAAGAAAAAAAAAAAAGATCAATGAATATATCAT-GATGAGCCGTGATGACCC	
18519	TCCGTCAGTATTCGAGGC	Diagnostic PCR for *Se*YCL036W integrations
18520	TCTTGGAACCTATCCTGGC	

pUD1298 as template, respectively. The *ScFAS2*, and *ScFAS2*^{G1250S} repair fragments were obtained by PCR amplification using primer pair 19763 & 19764, which incorporate compatible flanks with the *Se*YCL049C site (Bennis *et al.*, 2023), and pUD1299 and pUD1300 as template, respectively. Single *ScFAS* gene integrations were performed by co-transforming the respective *ScFAS1* repair fragment together with pUDP273 encoding the gRNA targeting *Sc*YCR087C, or the respective *ScFAS2* repair fragment together with pUDP269 encoding the gRNA targeting SeYCL049C into CBS 1483, resulting in IMI541 (*ScFAS1*), IMI542 (*ScFAS1*^{I306A}), IMI543 (*ScFAS1*^{R1834K}), IMI544 (*ScFAS1*^{I306A,R1834K}), IMI545 (*ScFAS2*) and IMI546 (*ScFAS2*^{G1250S}). To construct strains with both, *ScFAS1* and *ScFAS2* genes, the strains IMI545 (*ScFAS2*) and IMI546 (*ScFAS2*^{G1250S}) were co-transformed with pUDP273 and a respective *ScFAS1* repair fragment, resulting in the strains IMI547 (*ScFAS1 ScFAS2*), IMI548 (*ScFAS1*^{I306A} *ScFAS2*), IMI549 (*ScFAS1*^{R1834K} *ScFAS2*), IMI550 (*ScFAS1*^{I306A,R1834K} *ScFAS2*), IMI551 (*ScFAS1 ScFAS2*^{G1250S}), IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}), IMI553 (*ScFAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}).

The expression cassette for overexpression of *ScEEB1* was amplified from pUD1325 using primers 18517 & 18518 adding compatible flanks for *Se*YCL036W for genomic integrations. The PCR amplified fragment was co-transformed with pUDP270 (Bennis *et al.*, 2023) into IMI554 and CBS 1483, resulting in strains IMI577 and IMI578, respectively.

Yeast genomic DNA for diagnostic purposes was isolated using the lithium acetate-SDS method (Lõoke *et al.*, 2011). Gene integrations in *Se*YLC049C were confirmed with primers 18514 & 18515, *Se*YCL036W with 18519 & 18520, and *Sc*YCR087C with 18534 & 18535 (Bennis *et al.*, 2023).

Whole genome sequencing

Yeast genomic DNA of IMI551, IMI552, IMI553, IMI554 and IMI577 was isolated using the QIAGEN Genomic-tip 100/G kit (Qiagen, Hilden, Germany), following manufacturer's instructions. Genomic DNA concentrations were measured with the BR ds DNA kit (Invitrogen, Carlsbad, CA) using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The DNA quality was verified on the TapeStation System 4150 (Agilent). In-house nanopore sequencing was performed for strain IMI551, IMI552, IMI553 and IMI554 using a MinION sequencer (Oxford Nanopore Technologies, Oxford, United Kingdom) on a R10.4 flow cell (ONT). The sequencing library for MinION sequencing was prepared using the native barcoding sequencing kit SQK-NBD114.24 (ONT), allowing multiplexing of the four samples on one flow cell. Data acquisition from the MinION sequencer was performed using the MINKNOW software (ONT, version 23.04.6). Guppy (ONT, version 6.5.7GPU) was used for basecalling. Resulting .fastq files were filtered on length (> 1 kb) and mapped to a CBS 1483 derived reference strain with *in silico* added *ScFAS1*, *ScFAS2* (mutants) and *ScEEB1* sequences using Minimap2 (Li, 2018).

Illumina sequencing of strains IMI551, IMI552, IMI553, IMI554 and IMI577 was performed on a NovaSeqX 10B system (Macrogen-Europe, The Netherlands). The libraries were prepared using a PCR-free TruSeq kit (350 bp insert size and 150 bp paired end). The sequencing reads were mapped to the CBS 1483 with *ScFAS1*, *ScFAS2* (mutants) and *ScEEB1 in silico* constructed reference genome using the Burrows–Wheeler Alignment (BWA) tool (version 0.7.15) (Li & Durbin, 2009) and further processed using SAMtools (version 1.3.1) (Li *et al.*, 2009) (Tabel S5.1). Chromosomal copy number was estimated by the Magnolya algorithm (Nijkamp *et al.*, 2012) (Tabel S5.2). The sequences analyzed by visualizing the .bam files in the Integrative Genomics Viewer (IGV) software (version 2.8.9) (Thorvaldsdóttir *et al.*, 2013). All sequencing data (Tabel S5.1) are available at NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject accession number PRJNA1087702.

Fermentation in septum bottles

Frozen aliquots of CBS 1483, IMI541-IMI554, IMI577 and IMI578 were inoculated in 20 mL of YPD media in 50 mL Greiner tubes with air vents at 20 °C shaking at 200 rpm. Wake-up cultures were transferred to precultures in 100 mL YPD in 500 mL shake flasks grown at 20 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 100 mL bottles sealed with rubber stopper septum containing 60 mL full malt wort at 5.7 °P for strains CBS 1483, IMI541-IMI554, IMI577 and IMI578 at a starting OD_{660} of 0.5 mL^{-1}. The cultures were incubated at 20 °C and at 200 rpm for five days with regular daily sampling to determine cell density, sugar consumption, ethanol production, esters and ketones concentrations throughout the fermentation.

Fermentation in laboratory scale mini European Brewing Convention (E.B.C.) tubes

Frozen aliquots of CBS 1483, IMI541-IMI554, IMI577 and IMI578 were inoculated in 20 mL of YPD media in 50 mL Greiner tubes with air vents at 12 °C shaking at 200 rpm. Wake-up cultures were transferred to precultures in 100 mL YP maltose (YPM) (6%) in 500 mL shake flasks grown at 12 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 250 mL wort (17 °P) to obtain a starting cell density of approximately 5×10^6 cells mL^{-1}. Then, 80 mL of the prepared inoculum was transferred into laboratory scale tubes with a diameter/length ratio akin to tall E.B.C. tubes (Enari, 1977) in duplicate. The laboratory scale E.B.C tubes were equipped with Ankom system gas monitors for online CO_2 measurements (Ankom, Macedon NY), which were set to release pressure at 100 mbar and recording interval to 30 seconds. The fermentations were performed at 12 °C in a stationary incubator. After 14 days the fermentation was stopped and the supernatant was collected for analytical measurements including sugar, ethanol, ester and ketone concentrations and fatty acid extractions.

Fermentation in tall E.B.C. tubes

Frozen aliquots of CBS 1483, IMI552-IMI554 were inoculated in 100 mL of YPD media in 500 mL shake flasks at 12 °C shaking at 200 rpm. Wake-up cultures were transferred to precultures in four times 100 mL YPM (6%) in 500 mL shake flasks grown at 12 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 5 L wort (17 °P) to obtain a starting cell density of approximately 3.5×10^6 cells mL^{-1}. Then, 2.25 L of the prepared inoculum was transferred into the tall tubes in duplicate. The tall tubes were equipped with Bronkhorst EL-FLOW® Prestige mass flow meters (Bronkhorst, The Netherlands) for online CO_2 measurements. The fermentations were performed at 12 °C using cryostats for cooling. Samples for cell counts, °Plato, pH, sugar, ethanol and flavour compounds concentration determinations were taken daily. Cell count and viability were determined using a NucleoCounter YC-100™ (Chemometec A/S, Allerod, Denmark) according to the manufacturer's recommendations. Wort density was measured using an Anton Paar DMA35 portable density meter according to the manufacturer's recommendations. After 17 days the fermentation was stopped and the beer was collected by filtering the fermentation broth using Nalgene™ Rapid-Flow™ bottle top (45 mm, 0.2 µm) (Thermo Fisher Scientific). The beer was diluted to 5% ABV using carbonated water and stored in brown-glass beer bottles at 4 °C.

Fatty acid extraction

Fatty acids in supernatant were analysed as methyl-ester derivatives by gas chromatography with flame-ionization detection (GC-FID). Supernatant was aliquoted in 10 mL portions in 15 mL Greiner tubes and to each aliquot 100 µL of internal standard solution (30 mg heptanoic acid in 15 mL hexane) was added. For acidification, 1 mL 1 M HCl was added, followed by addition of 2.5 mL of a mixture of equal amounts (v/v) of

methanol and chloroform and 5 min vigorously shaking of the sample. The sample was spun down at 2000 rcf for 10 min and the chloroform layer was transferred to a glass trans-methylation tube with a glass Pasteur pipet. The chloroform was evaporated under nitrogen gas flow. For methylation, 1.5 mL methanol, 0.3 mL HCL solution (8% w/v solution made from 9.7 mL of concentrated HCl and 41.5 mL of methanol) and 1 mL hexane were added in the respective order. The mixture was vortexed for 1 min and heated to 80 °C for 1 hour with vortexing the tubes briefly every 15 min. Thereafter, the samples were cooled on ice and 1 mL water was added and vortexed. To separate the organic and aqueous phase, the sample was spun down for 5 min at 1900 rcf. The organic layer was transferred into a GC vial using a Pasteur pipet and loaded onto the GC.

Analytical measurements

Sugars (glucose, fructose, maltose, maltotriose) and ethanol concentrations were measured using HPLC (Agilent Technologies, 1260 HPLC system) equipped with a Bio-Rad HPX-42A column (300 × 7.8 mm, 25 micron) (Bio-Rad) and an 1260 Refractive Index Detector (RID). The column was operated at 75 °C, and the elution was performed using milliQ at 0.5 mL min^{-1} for 30 min. Compounds were measured using a RID at 35 °C. Samples were 0.2 µm filter-sterilized before analysis.

Vicinal diketone (diacetyl, 2,3-pentadione) concentrations were measured using static headspace gas chromatography in a 7890A Agilent GC (Agilent) with an electron capture detector on a CP-Sil 8 CB (50 m x 530 µm x 1 µm) capillary column. 0.5 mL of each sample was heated up to 65 °C for 30 min prior to injection. A split flow of 8 mL N_2 min^{-1} was used, with a split ratio of 1:1. The injector temperature was set at 120 °C. The oven was set at 35 °C for 3 minutes, followed by a 10 °C min^{-1} ramp to 95 °C. The ECD temperature was set at 150 °C with a make-up flow of 30 ml N_2 min^{-1}.

Higher alcohols and esters were analysed using static headspace gas chromatography (GC) with a flame ionization detector FID (Agilent technologies 7890A) and a DB-WAXetr capillary column (30 m x 320 µm x 1 µm). 2.5 mL samples were heated up to 50 °C for 5 min using a CTC Combi Pal headspace auto-injector prior to the injection. A split flow of 9.88 mL N_2 min^{-1} was used, with a split ratio of 5:8:1. The temperature of the injector was 250 °C, with a flow of 10 mL N_2 min^{-1}. The oven temperature profile started at 55 °C followed by an increase of 20 °C min^{-1} to 160 °C with a hold time of 4.75 min. The FID temperature was set at 250 °C with a make-up flow of 10 ml N_2 min^{-1}.

Fatty acid methyl esters were analysed on an Agilent Technologies 7890A GCFID system equipped with an FID-1000–220 Gas Station (Parker Balston, Haverhill, MA) and an Agilent Technologies 7693 Autosampler. A VF-5 ms column (30 m x 25 µm, 0.25 µm, Agilent part no. CP9013) was used for separation, and H_2 was used as carrier gas at a constant flow of 25 mL min^{-1}. 1 µL sample was injected at a split ratio of 10:1. The oven

temperature was initially set at 50 °C and held for 4 minutes and then increased to 180 °C at a rate of 10 °C min^{-1}. Inlet temperature was set at 150 °C, and FID temperature at 330 °C. The Supelco FAME mix #7 C6-C10 (VWR International B.V, Amsterdam, The Netherlands) was used to calibrate the GC-FID system for quantification of individual fatty acid methyl esters. Data was corrected using the internal standard concentrations.

Sensory analysis

The institutional Human Research Ethics Committee (HREC) approved the tasting session under ID3948. Sensory analysis of the brewed beer was conducted internally by a tasting panel of 14 participants who had received prior sensory training. All participants consented verbally after being informed about the study's details. An attribute list to described odour, taste, flavour and mouthfeel was composed. The attribute list served to evaluate the sensory difference of the samples, beers produced with strains IMI552, IMI553, IMS554 compared to the control beer produced by the strain CBS 1483. The participants could identify different attributes between a sample and the control and score the difference with + or – with plus denoting a higher attribute intensity and minus a lower attribute intensity relative to the control. For the evaluation, 25 ml of each sample was served in beer glasses. The samples were anonymized and served at 4 °C and examined at room temperature. The sensory data were collected manually. The tasting session was not replicated.

Data analysis and statistics

GraphPad Prism software (GraphPad Software version 10.3) was used to perform statistical analysis and data visualization. Data were analysed by one-way (bar plots) or two-way (time-course) ANOVA, followed by Tukey's multiple comparisons tests. Significance is indicated as * $p < 0.01$ and ** $p < 0.0001$, unless indicated otherwise.

5.3. Results

Single *FAS* mutant overexpression resulted in specific ethyl ester production profiles

To study how mutations in the FAS complex affect ethyl ester level in lager brewing yeast fermentation, mutant alleles of *ScFAS1* and *ScFAS2* were expressed alongside their non-mutated counterparts in *S. pastorianus* CBS 1483. For this, alleles encoding non-synonymous mutations I306A and R1384K in ScFas1 and G1250S in ScFas2 were introduced in landing sites *Sc*YCR087C and *Se*YCL049C located on *Se*ScCHRIII which counts four copies, respectively using CRISPR-Cas9 genome editing (Bennis *et al.*, 2023) (Figure 5.1B, Figure S5.1). As such, a strain set including IMI542 (Δ*Sc*YCR087C::*ScFAS1*^{I306A}), IMI543 (Δ*Sc*YCR087C::*ScFAS1*^{R1834K}), IMI544 (Δ*Sc*YCR087C::*ScFAS1*^{I306A,R1834K}), IMI546 (Δ*Se*YCL049C::*ScFAS2*^{G1250S}) was constructed. Control strains carrying an extra *ScFAS1* or *ScFAS2* integration IMI541 (Δ*Sc*YCR087C::*ScFAS1*) and IMI545 (Δ*Se*YCL049C::*ScFAS2*) were also constructed. To prevent imbalance in expression of these extra *ScFAS1* and *ScFAS2* alleles, they were cloned under the control of the same regulatory sequences, the *ScTEF1* promoter and *ScCYC1* terminator (Table 5.2, Supplementary information).

The strains IMI541-546 were evaluated for ethyl esters production using 5.7 °P full malt wort at 20 °C. The strains were grown in bottles sealed with rubber stoppers to maintain anaerobic conditions characteristic of brewing fermentation, but it is also known that ethyl ester synthesis is notably low in this type of cultivation format probably caused by the building CO_2 concentration. To illustrate this, the reference *S. pastorianus* strain CBS 1483 neither produced detectable ethyl hexanoate nor ethyl octanoate under these conditions. Overexpression of wild type *ScFAS2* (IMI545) displayed a similar phenotype (Figure 5.2, Figure S5.2) while overexpression of *ScFAS1* (IMI541) resulted in 0.07 ± 0.00 mg L^{-1} of ethyl octanoate (Furukawa *et al.*, 2003) (Figure 5.2BC, Figure S5.2). Conversely, expression of both mutant alleles resulted in significant increase of ethyl ethers (Figure 5.2BC, Figure S5.2). The expression of the *ScFAS1*^{I306A} mutation (IMI542), located in the AT domain causing elevated uptake of acetyl forcing the start of a new fatty acid synthesis cycle, specifically increased ethyl hexanoate production to reach 0.10 ± 0.00 mg L^{-1} ($p < 0.0001$), while expression of *ScFAS1*^{R1834K} (IMI543), located in the MPT domain and lowering the affinity for malonyl moiety, increased ethyl octanoate production up to 0.15 ± 0.00 mg L^{-1} ($p < 0.0001$) (Figure 5.2BC, Figure S5.2). The combination of these mutations led to production of both ethyl hexanoate and ethyl octanoate. The strain IMI544 (*ScFAS1*^{I306A,R1834K}) produced 0.09 ± 0.00 mg L^{-1} ($p < 0.0001$) of ethyl hexanoate and 0.095 ± 0.01 mg L^{-1} of ethyl octanoate ($p < 0.0001$) demonstrating that the expression of the double mutation in *FAS1* was additive as in contrast to the single mutants *ScFAS1*^{I306A} and *ScFAS1*^{R1834K} that led to increase in ethyl hexanoate and ethyl octanoate respectively, where the double mutant of *FAS1* (*FAS1*^{I306A,R1834K}) combined these improvements. The expression of the *ScFAS2*^{G1250S} mutation, preventing short chain intermediates from entering the KS

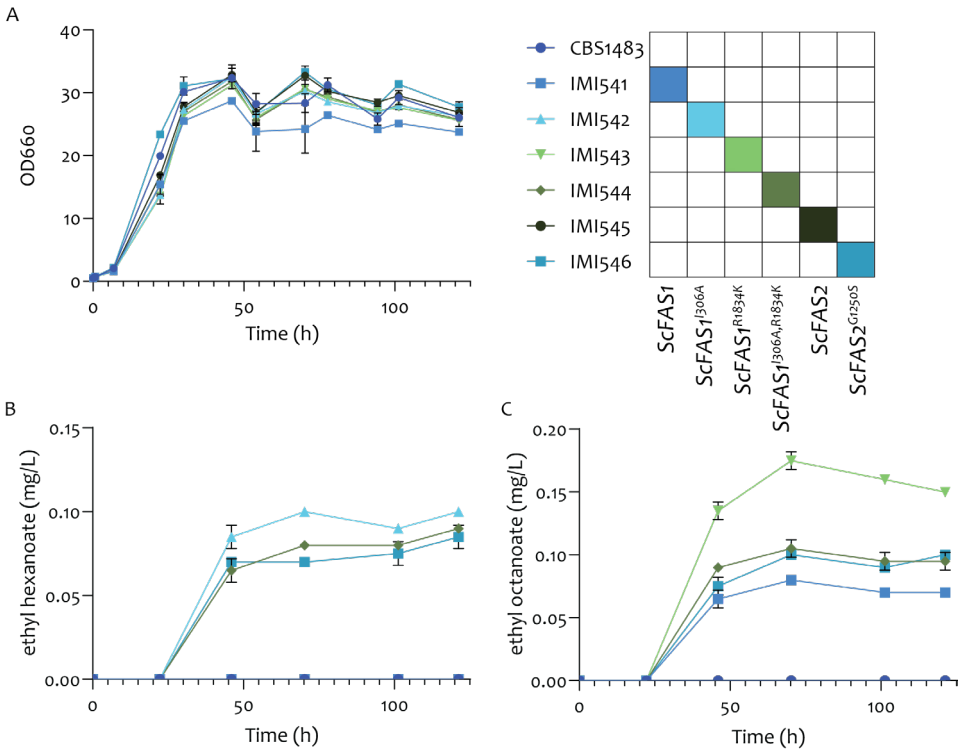


Figure 5.2. Evaluation of single *ScFAS1* and *ScFAS2* mutants in CBS 1483 on ethyl ester biosynthesis. Growth and ethyl ester profiles of septum flasks fermentation (5.7 °P, 20 °C, 200 rpm) with engineered strains IMI541-IMI546 and parental strain CBS 1483 were monitored during fermentation septum flasks on 5.7 °P wort and cultivated at 20 °C shaking at 200 rpm. **A)** Growth, **B)** ethyl hexanoate and **C)** ethyl octanoate profiles were measured.

domain, favoured ethyl hexanoate production over ethyl octanoate. The strain IMI546 (*ScFAS2*^{G1250S}) resulted in production of ethyl hexanoate (0.085 mg L^{-1}, $p < 0.0001$) and ethyl octanoate (0.10 mg L^{-1}, $p < 0.0001$) (Figure 5.2BC, Figure S5.2). These mutations enhanced ethyl ester concentrations without impacting growth behaviour (Figure 5.2A, Figure S5.3), aligning with expected changes in fatty acid levels and offering potential for tailored ethyl ester profiles.

Combinations of *ScFAS1* and *ScFAS2* mutations resulted in a synergistic increase in ethyl ester production.

To investigate the combined effects of mutations in the FAS complex on ethyl ester production, strains IMI545 (Δ*Se*YCL049C::*ScFAS2*) and IMI546 (Δ*Se*YCL049C::*ScFAS2*^{G1250S}) were used as host strains for integrating the *ScFAS1* variants at the *Sc*YCR087C genomic location (Figure 5.1B, Figure 5.3, Figure S5.1). Similar to single overexpression of native *ScFAS1*, overexpression of both native *ScFAS1* and *ScFAS2* genes (IMI547 (Δ*Se*YCL049C::*ScFAS2* Δ*Sc*YCR087C::*ScFAS1*)) resulted in

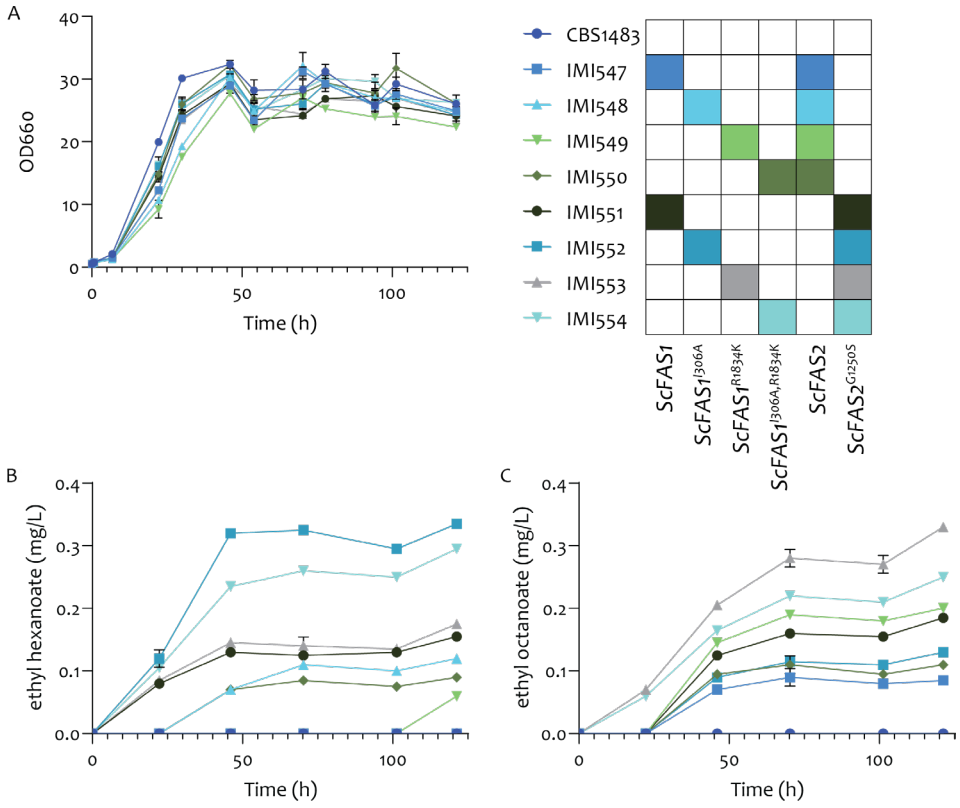


Figure 5.3. Evaluation of the combinatorial effect of *ScFAS1* and *ScFAS2* mutants in CBS 1483 on ethyl ester biosynthesis. Growth and ethyl ester profiles of septum flasks fermentation (5.7 °P, 20 °C, 200 rpm) with engineered strains IMI547-IMI554 expressing combinations of *ScFAS1* and *ScFAS2* mutant genes and the parental strain CBS 1483 were monitored during fermentation septum flasks on 5.7 °P wort and cultivated at 20 °C shaking at 200 rpm. **A)** Growth, **B)** ethyl hexanoate and **C)** ethyl octanoate profiles were measured.

the production of ethyl octanoate, confirming the contribution of Fas1 to increased production of C8 fatty acid and its ethyl ester derivative (Figure 5.3C, Figure S5.2). Overexpression of *ScFAS1*^{I306A} and/or *ScFAS1*^{R1834K} combined with overexpression of the *ScFAS2* (IMI548, 549 and 550) resulted in similar ethyl ester production profiles compared to strains without *ScFAS2* overexpression (IMI542, 543 and 544) (Figure 5.3BC, Figure S5.2). The combination of *ScFAS1*^{I306A} (IMI548) or *ScFAS1*^{R1834K} (IMI549) with *ScFAS2* resulted in 20% ethyl hexanoate and in 33% ethyl octanoate increase respectively relative to the single *ScFAS1* mutants IMI542 and IMI543. Overexpression of *ScFAS1*^{I306A,R1834K} together with *ScFAS2* (IMI550) resulted in similar production profiles (0.09 ± 0.00 mg L^{-1} ethyl hexanoate and 0.11 ± 0.00 mg L^{-1} ethyl octanoate, $p > 0.01$) as single *ScFAS1*^{I306A,R1834K} overexpression (Figure 5.3BC, Figure S5.2).

Combining the *ScFAS1* variants with overexpression of *ScFAS2*^{G1250S} resulted in a systematic increase in the total ethyl ester production demonstrating the relevant role of the *ScFAS2*^{G1250S} mutation. This synergistic effect was obvious when comparing the performance of IMI542 (*ScFAS1*^{I306A}) and IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}). IMI552 exhibited a 3.4-fold significant higher ethyl hexanoate concentration. Additionally, IMI552 was also capable of ethyl octanoate concentration up to 0.13 ± 0.00 mg L^{-1} (Figure 5.3C, Figure S5.2). Similarly, the combination of *ScFAS2*^{G1250S} and *ScFAS1*^{R1834K} (IMI553) resulted in the highest concentration of ethyl octanoate (0.33 ± 0.00 mg L^{-1}, $p < 0.0001$ compared to IMI543 and IMI546), and high concentration of ethyl hexanoate (0.18 ± 0.01 mg L^{-1}). Combining all three mutations (IMI554, *ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}) did not further increase ethyl ester synthesis but still resulted in second highest final concentrations of the ethyl esters (0.30 ± 0.01 mg L^{-1} of ethyl hexanoate and 0.25 ± 0.00 mg L^{-1} of ethyl octanoate, $p < 0.0001$ relative to IMI544 and IMI546) (Figure 5.3BC, Figure S5.2). Growth and sugar metabolism remained similar to the control strain (Figure 5.3A, Figure S5.4) results demonstrate that the combination of these mutations have a direct, synergistic effect on the ethyl ester production and can produce a broad range of flavour profiles.

Characterization of ethyl ester producing strains in laboratory scale mini E.B.C. tubes

In the exploration of flavour molecule production during fermentation, the design of brewing reactors holds paramount importance. To emulate brewing conditions on a laboratory scale, mini tubes with a diameter/length ratio akin to E.B.C. tubes (Enari, 1977) were employed for lab-scale fermentations. These tubes were inoculated with engineered strains IMI541 to IMI554 alongside the parental strain CBS 1483 in 17 °P wort and statically incubated at 12 °C while meticulously monitoring the cumulative pressure. Following a 14-day incubation period, the supernatant of these mini tubes was collected and subjected to analysis. Notably, strains IMI551-IMI554, expressing the *ScFAS2*^{G1250S} allele combined with the native *ScFAS1* gene, exhibited remarkable performance (Figure 5.4). Ethyl ester concentrations were more than quadrupled for ethyl hexanoate and doubled for ethyl octanoate and ethyl decanoate (Figure 5.4ABC) relative to CBS 1483. Ethyl hexanoate concentration increased to 1.94 ± 0.07 mg L^{-1} in strain IMI552, specialised for C_6 acyl chain ester production, compared to 0.36 ± 0.03 mg L^{-1} for the parental strain CBS 1483. Similarly, strain IMI553, tailored for C_8 acyl chain ester synthesis, yielded 1.03 ± 0.18 mg L^{-1} ethyl octanoate versus 0.50 ± 0.06 mg L^{-1} for CBS 1483. Strain IMI554, encompassing all three examined *ScFAS* mutations (*ScFAS1*^{I306A,R1834K} and *ScFAS2*^{G1250S}), produced 1.62 ± 0.04 mg L^{-1} and 0.93 ± 0.01 mg L^{-1} ethyl hexanoate and ethyl octanoate, respectively (Figure 5.4ABC). While ethyl decanoate concentration fell below detection limit during septum flask cultivation, mini tube fermentations led to a significant rise in ethyl decanoate concentration for the engineered strains compared to the parental strain CBS 1483 (Figure 5.4ABC).

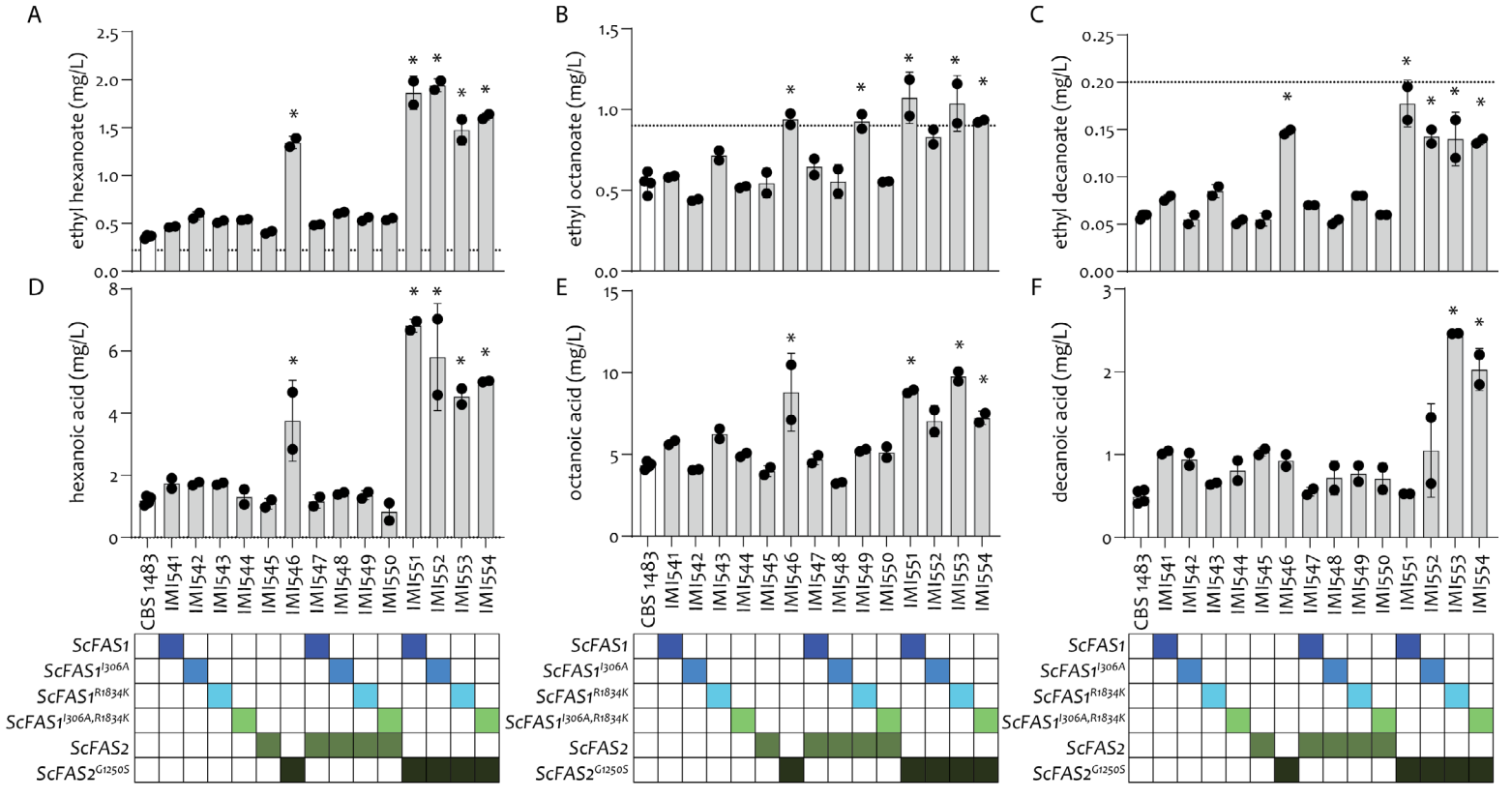


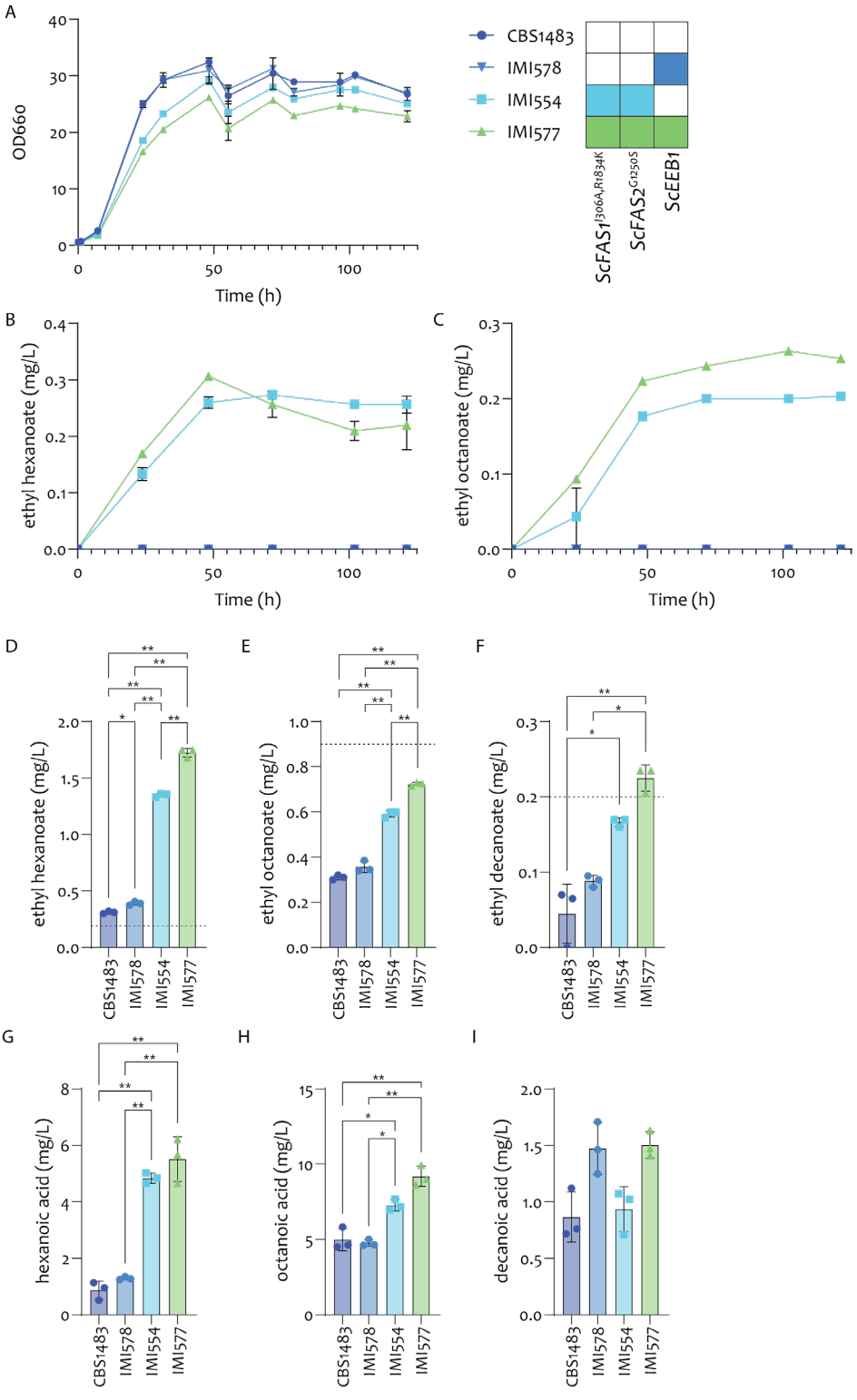
Figure 5.4. Ethyl ester and fatty acid production of *ScFAS1* and *ScFAS2* engineered strains during lab scale E.B.C. tube fermentations. Ethyl esters **(A,B,C)** and fatty acid **(D,E,F)** measurements of stationary laboratory scale E.B.C. tube fermentations (17 °P, 12 °C) with engineered strains IMI541-IMI554 and parental strain CBS 1483. The dashed line indicated the human sensory threshold levels for the respective ethyl esters. (* $p < 0.005$ compared to CBS 1483).

Furthermore, an investigation into whether fatty acid intermediates accumulate in the engineered strains as a consequence of the incorporated mutations was conducted. Particularly, strains IMI551-IMI554, containing *ScFAS2*^{G1250S} and one of the *ScFAS1* variants, exhibited a significant increase in hexanoic acid excretion, paralleling the fold-change observed in the ethyl ester derivative (Figure 5.4DEF). Overall, the ethyl ester concentration exhibited a correlation with the fatty acid concentration in the supernatant, indicating equilibrium in the reaction.

Enhanced ethyl ester production via combined expression of *ScFAS* mutants and acyl-coenzymeA:ethanol O-acyltransferase esterase *ScEEB1*

The extracellular concentrations of fatty acids markedly increased upon expression of combinations of *ScFAS1* and *ScFAS2* mutations (Figure 5.4) suggesting that in these strains, the limitation shifted from MCFA supply by the FAS complex to the esterification reaction catalysed by the AEAT enzymes encoded by *EHT1* and *EEB1*. We hypothesized that with the increased production of acyl-CoA intermediates caused by *ScFAS1* and *ScFAS2* mutants expression, overexpressing *ScEEB1* could divert the flux from the elevated levels of acyl-CoA intermediates to ethyl esters and potentially lower extracellular fatty acid concentrations. *S. pastorianus* CBS 1483 genome harbours two *EEB1* alleles one from *S. cerevisiae* and another from *S. eubayanus* subgenomes. These two alleles shared an identity of 77% at DNA and 83% at amino acid level.

Thus, to test this hypothesis, the *ScEEB1* gene was integrated into CBS 1483 and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}) at the *Se*YCL036W genomic location (Bennis *et al.*, 2023), resulting in strains IMI577 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S} *ScEEB1*) and IMI578 (*ScEEB1*). Solely overexpressing the main esterase encoded by *ScEEB1* (IMI578) did not notably enhance ethyl ester production in septum flask cultivation (5.7 °P full malt wort at 20 °C) (Figure 5.5ABC, Figure 5.5). However, in combination with *ScFAS1*^{I306A,R1834K} and *ScFAS2*^{G1250S} genes with *ScEEB1* resulted in a significant increase in ethyl octanoate production (0.20 ± 0.01 mg L^{-1} for IMI554 versus 0.25 ± 0.01 mg L^{-1} for IMI577, $p < 0.01$), while the ethyl hexanoate concentration was not significantly affected under these conditions (Figure 5.5BC). In lab-scale E.B.C. mini tubes, IMI577 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S} *ScEEB1*) exhibited a 28%, 22% and 35% increase in ethyl hexanoate, ethyl octanoate and ethyl decanoate respectively relative to IMI554 (Figure 5.5DEF), reaching levels above sensory thresholds for the C_6 and C_{10} esters. In the meantime, expression of *ScEEB1* was accompanied by an increase in C_6, C_8 and C_{10} carboxylic acids (Figure 5.5GHI). These results suggest that despite having a positive impact on ethyl ester profiles, the expression of *ScEEB1* was not sufficient or not well balanced with the FAS activity to reduce fatty acid concentrations.


A
OD660
Time (h)
CBS1483
IMI578
IMI554
IMI577
ScFAS1I306A,R1834K
ScFAS2G1250S
ScEEB1
B
ethyl hexanoate (mg/L)
Time (h)
C
ethyl octanoate (mg/L)
Time (h)
D
ethyl hexanoate (mg/L)
E
ethyl octanoate (mg/L)
F
ethyl decanoate (mg/L)
G
hexanoic acid (mg/L)
H
octanoic acid (mg/L)
I
decanoic acid (mg/L)

Towards large scale brewing fermentations: *S. pastorianus* strain assessment in Tall E.B.C. tubes

The cultivation standard to predict and evaluate the stability of brewing characteristics of a lager yeast strain is the E.B.C. tall tube (Enari, 1977), relatively high liquid column fermenter of approximately 2L with dimension ratio close to industrial fermentation equipment. Thus, to more accurately predict the suitability of the engineered strains for producing proper ethyl ester flavour profiles at larger scale, the strains IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}), IMI553 (*ScFAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}) were cultivated on 17 °P full malt wort in 2.25 L E.B.C. tall tubes operating at 12 °C.

The ethyl ester profile was notably enhanced relative to the parental strain CBS 1483. Specifically, IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}) exhibited a significant increase in ethyl hexanoate production, yielding 2.92 ± 0.02 mg L^{-1} compared to 0.56 ± 0.01 mg L^{-1} produced by CBS 1483 ($p < 0.0001$) (Figure 5.6A). Additionally, both IMI553 (*ScFAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}) also showed significantly higher ethyl hexanoate production (1.88 ± 0.02 mg L^{-1} and 2.00 ± 0.01 mg L^{-1}, respectively, $p < 0.0001$) compared to CBS 1483 (Figure 5.6). IMI552 and IMI553 produced the largest amount of ethyl octanoate (1.12 ± 0.01 mg L^{-1} and 1.06 ± 0.01 mg L^{-1}), representing a 1.42- and 1.24-fold higher concentration than that of CBS 1483 (0.85 ± 0.00 mg L^{-1}) (Figure 5.6B). Moreover, ethyl decanoate levels in the engineered strains were all significantly higher than in CBS 1483 (0.09 ± 0.00 mg L^{-1}, $p < 0.0001$). IMI553 achieved the highest ethyl decanoate concentrations (0.30 ± 0.01 mg L^{-1}), followed by IMI554 (0.24 ± 0.01 m L^{-1}) and IMI552 (0.16 ± 0.01 mg L^{-1}) (Figure 5.6C). The engineering of the FAS complex did not alter sugar consumption and ethanol production profiles (Figure S5.6, Figure S5.7). Similarly, profiles of other volatile components (e.g. diacetyl, 2,3-pentadione, isobutanol, isoamyl alcohol) remained unaffected (Figure S5.7, Figure S5.8). This was contrasting with two phenotypes: i) Remarkably, throughout fermentation, the cell counts of strains expressing *ScFAS* mutant genes were lower relative to *S. pastorianus* CBS 1483 and was also accompanied by a lower cell viability near the end of fermentation. The viability of the engineered strains ranged from 89% to 93% when that of CBS 1483 never dropped below 96% (Figure S5.5). ii) The level of acetate esters (ethyl acetate, ethyl butyrate, isoamyl acetate, and isobutyl acetate) were consistently lower in the engineered strains IMI552-IMI554 than in CBS 1483 (Figure S5.8). In contrast, the concentration profiles of higher alcohols and vicinal diketones were not affected (Figure S5.9 and Figure S5.10).

Figure 5.5. Overexpression of *ScEEB1* increases ethyl ester concentrations in *FAS* mutant strains, but does not affect the fatty acid concentrations. **A)** Growth, **B)** ethyl hexanoate and **C)** ethyl octanoate concentrations of septum flasks fermentation (5.7 °P, 20 °C, 200 rpm) with engineered strains IMI578, IMI554 and IMI577 and parental strain CBS 1483. The strains were also cultivated in stationary lab scale E.B.C. tubes (17 °P, 12 °C). After 14 days of cultivation, the ethyl esters **(D,E,F)** and respective fatty acid **(G,H,I)** concentrations were measured (* $p < 0.01$, ** $p < 0.0001$).

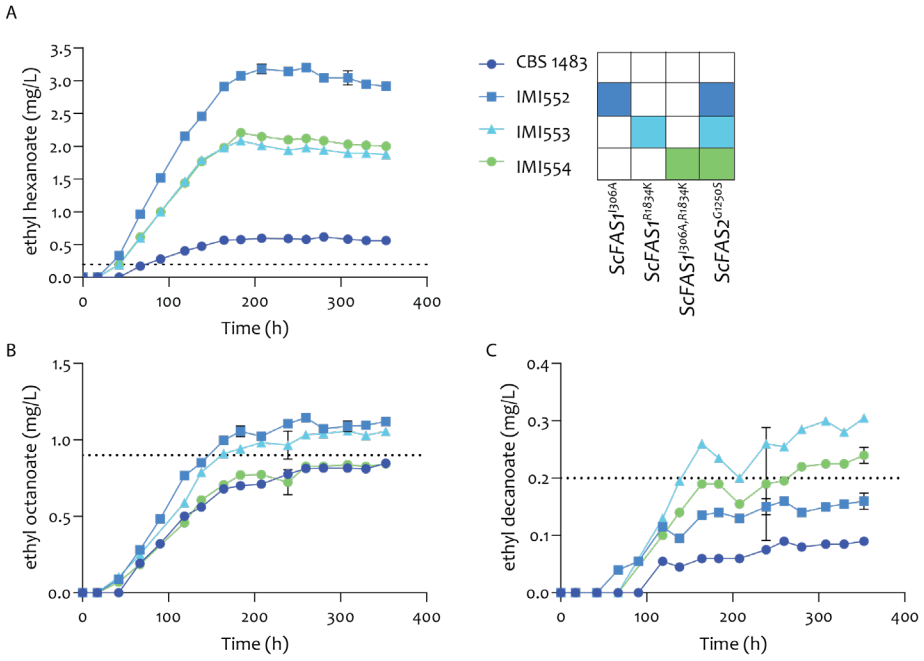


Figure 5.6. Ethyl ester profiles of CBS 1483 (—●—), IMI552 (—■—), IMI553 (—▲—) and IMI554 (—●—) cultivated in tall tube fermentations. **A)** Ethyl hexanoate, **B)** ethyl octanoate and **C)** ethyl decanoate profiles of CBS 1483, IMI552, IMI553 and IMI554 cultivated during 17 days in stationary tall tube fermentations (17 °P, 12 °C).

Sensory analysis of beers produced with IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}), IMI553 (*ScFAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S})

At the end of E.B.C.-tall tube fermentations, the contents of the duplicate fermenters were mixed and subsequently subjected to filtration through a 0.2 µm filtration membrane. The resulting filtrate was then diluted to an alcohol by volume (ABV) concentration of 5% with carbonated water and bottled. Following a 14-day storage period at 4°C, the four produced beers underwent sensory analysis conducted by a panel comprising of 14 trained tasters. The three beers brewed with the engineered strains manifested distinguishable characteristics from the beer brewed with *S. pastorianus* CBS 1483. Notably, they showcased heightened aroma intensities attributed to the presence of ethyl esters. Specifically, notes reminiscent of pineapple and aniseed, indicative of ethyl hexanoate and ethyl octanoate, respectively, were more pronounced in beers fermented by IMI552, IMI53, and IMI554 than in the CBS 1483 beer (Figure 5.7). Additionally, IMI552 exhibited a distinct red fruit note, an aroma determinant often associated with ethyl hexanoate. This observation correlated with the measured level of ethyl hexanoate in IMI552 fermentation (Figure 5.6A). However, these positive sensory attributes were counterbalanced by flavours associated with

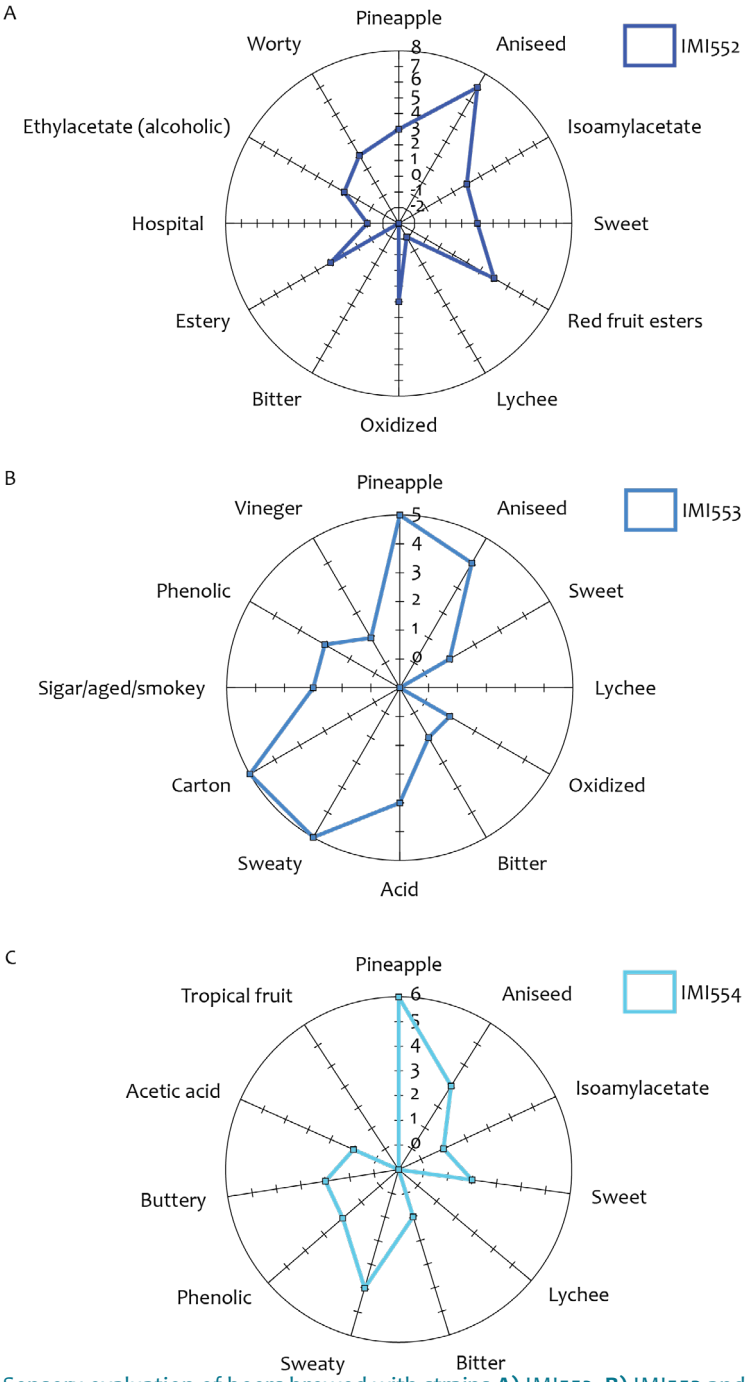


Figure 5.7. Sensory evaluation of beers brewed with strains **A)** IMI552, **B)** IMI553 and **C)** IMI554 compared to the control strain CBS 1483. After 17 days of cultivation in stationary tall tube fermentations (17 °P, 12 °C), the fermentation broth was collected, the yeast was filtered out, the beer was normalized to 5% ABV (36.6 g L^{-1} ethanol) using carbonated water and stored in brown bottles at 4 °C. A panel consisting of fourteen trained accessors with a 'difference to control' analysis.

MCFA. Flavours such as sweaty, carton-like, acidic, and acetic acid were detected in beers brewed with the engineered strains. Notably, the sweaty flavour identified in IMI553 and IMI554 is characteristic of hexanoic acid (Figure 5.7), also known as caproate, with its etymological roots derived from "capra", Latin for goat, due to its resemblance to the scent of goat's milk, which is rich in this carboxylic acid.

Collectively, these findings underscore that elevated levels of ethyl esters resulting from yeast biosynthesis during fermentation contribute to pineapple and aniseed aromas in the finished beer. However, the presence of aroma determinants derived from MCFA may exert an influence on the overall taste profile.

5.4. Discussion

The delicate balance of flavour components in beer profoundly influences its quality, as extreme concentrations of individual compounds or their synergistic interactions can detrimentally affect the overall flavour profile. Among these flavour-enhancing molecules, esters play a pivotal role despite their occurrence in relatively minute quantities (Verstrepen *et al.*, 2003a). While the research focus on acetate esters has been more pronounced, ethyl esters are equally important in shaping the profile of flavoured fermentation products.

Various methods can be employed to adjust ethyl ester levels, including augmenting acetyl-CoA supply or employing metabolic 'pulling' strategies involving the overexpression of ethanol-acyltransferase alone or in combination with a thioesterase. Regardless of the medium-chain fatty acid (MCFA) species involved, these approaches consistently resulted in elevated ethyl ester levels but without exceeding HST levels. In our efforts to enhance ethyl ester production in *S. pastorianus*, we focused on increasing the supply of acyl-CoA precursors. While several strategies have been explored to boost MCFA production in *S. cerevisiae* focussed on the esterase reaction (Fernandez-Moya & Da Silva, 2017, Wernig *et al.*, 2020), we chose to modify the fatty acid synthase (FAS) complex by incorporating three distinct mutations, each enabling a unique mechanism to stimulate premature MCFA release. These genetic alterations not only led to an overall increase in ethyl esters, but also allowed for precise adjustment for specific ethyl ester profiles. Our findings are consistent with prior research indicating that overexpression of the native *ScFAS1* gene enhances C_8-MCFA production (Furukawa *et al.*, 2003). Specifically, mutations I306A in *ScFAS1* and G1250S in *ScFAS2* were designed to enhance ethyl hexanoate production, with G1250S having the most significant effect. Conversely, the R1834K mutation in *ScFAS1* elevated ethyl octanoate production. These distinct genetic modifications enable fine-tuning of ethyl hexanoate and ethyl octanoate levels, facilitating flavour customization according to user preferences.

These profiles are ultimately controlled by the ability of these esters to diffuse outside the cell. The diffusion rate over the cell membrane decreases proportionally as the chain length increases, ranging from 100% for ethyl hexanoate to 8-17% for ethyl decanoate, while even longer-chain fatty acid ethyl esters tend to remain within the cell (Nykänen *et al.*, 1977, Suomalainen, 1981, Bardi *et al.*, 1998, Bardi *et al.*, 1999, Saerens *et al.*, 2008), which is matching with our observations on the reduced extracellular concentrations. Ethyl-hexanoate levels were in most engineered strains higher than ethyl-octanoate levels which were higher than ethyl-decanoate, attributing to the reduced membrane transport.

While the endeavour to enhance the contribution of ethyl esters to the taste and aroma profile of beer was deemed successful, sensory analysis of the fermentation products, coupled with medium-chain fatty acid (MCFA) analysis, revealed a significant increase in fatty acid concentration (up to 4-fold) in the supernatant of the engineered strain fermentations (Figure 5.4). This increase manifested in off-notes, including sweaty and cardboard-like flavours, characteristic of the presence of hexanoic, octanoic, and decanoic acids. Efforts to mitigate this side effect also known as "caprylic flavour" (Clapperton, 1978), by expressing an acyl-coenzymeA:ethanol O-acyltransferase esterase (*Sc*Eeb1) in strains harbouring variants of the FAS complex showed limited feat, as no significant reduction in fatty acid content of the beer was observed. Despite an increase in ethyl ester concentrations, the acyl-coenzymeA:ethanol O-acyltransferase esterase may still serve as the rate-limiting step for ethyl ester biosynthesis. In *S. cerevisiae ScEEB1* is not the only AEAT encoding gene, it harbours a paralog *ScEHT1* as well as the ethanol acetyltransferase encoded by *ScEAT1*, which alone or in combination could be tested to improve ethyl ester : MCFA ratio in the future.

Altering the aroma compound profile in beer necessitates maintaining a balance between the metabolites present in the final product within certain limits. This implies that strain engineering may not necessarily aim for high yield and titre of a single compounds as is common in regular metabolic engineering studies. The ratio between ethyl esters and MCFA is critical, as MCFA can be directly converted into ethyl esters. However, the link to other metabolites may be less apparent and as critical. For instance, strains combining mutations in *ScFAS1* and *ScFAS2* (IMI552, IMI553, and IMI554) not only exhibited higher ethyl ester biosynthesis rates, but also significantly lower levels of acetate esters (ethyl acetate, isoamyl acetate, and isobutyl acetate) (Figure S5.8), which also contribute to the final aromatic bouquet. These results might connect to potential competition for acetyl-CoA which serves as the precursor for acyl-CoA biosynthesis involved in ethyl ester formation, while also serving as a substrate for acetate esters. This suggests that future strain improvement strategies should embrace metabolic pathway balancing to avoid accumulation of unwanted intermediates (e.g. carboxylic acids) by investigating gene dosage, tuning transcriptional modulation or

applying post-translational variation based on for instance synthetic protein scaffolds to optimize and control metabolic fluxes.

Often regarded as non-genetically tractable, this study unequivocally demonstrates that *Saccharomyces pastorianus* can indeed undergo metabolic engineering approaches, enabling the prototyping of new lager brewing strains with customizable aroma profiles. However, precision genetic engineering remains more complex in lager yeasts than in laboratory haploid *S. cerevisiae* strains due to the challenge of conserving the ploidy of the engineered strain. Transformation may have a mutagenic effect by altering the ploidy of *S. pastorianus*. In this study, several engineered strains showed the loss of one or more chromosome copies (Tabel S5.2) (Gorter de Vries *et al.*, 2020). Recurrently, brewing-relevant phenotypic traits, such as the ability to consume maltotriose (e.g. strain IMI541, Figure S5.3), may be lost upon transformation. This necessitates screening for transformants that have retained the phenotypic trait (Bennis *et al.*, 2023). The reason behind this phenotype loss is still not understood. To ensure strain stability, the strain is transferred to new medium at least three times before sequencing. Further systematic deep-sequencing of engineered strains (e.g. IMI551-554) with illumia and nanopore technology did not reveal trace of recombination supporting genomic stability of engineered strains. In particular, redundancy of the *pTEF1* and *tCYC1* regulatory sequences used to control the expression of *FAS* alleles did not show chromosomal rearrangement of *Sc*Se*CHRIII*. Despite these potential pitfalls, these brewing phenotypic determinants would be exceedingly challenging to achieve through traditional strain improvement methods. Therefore, this approach holds significant promise for the development of novel lager brewing strains with enhanced or unique characteristics, potentially changing the brewing industry.

Acknowledgements

We are thankful to Prof. J.T. Pronk (Delft University of Technology), Dr. V.M. Boer and Mr T. Elink Schuurman for their support during this project.

Data availability

Supplementary information can be accessed via the online publication.

5.5. References

Aritomi K, Hirosawa I, Hoshida H, Shiigi M, Nishizawa Y, Kashiwagi S & Akada R (2004) Self-cloning yeast strains containing novel *FAS2* mutations produce a higher amount of ethyl caproate in Japanese sake. *Bioscience Biotechnology Biochemistry* **68**: 206-214.

Bardi L, Crivelli C & Marzona M (1998) Esterase activity and release of ethyl esters of medium-chain fatty acids by *Saccharomyces cerevisiae* during anaerobic growth. *Canadian Journal of Microbiology* **44**: 1171-1176.

Bardi L, Cocito C & Marzona M (1999) *Saccharomyces cerevisiae* cell fatty acid composition and release during fermentation without aeration and in absence of exogenous lipids. *International Journal of Food Microbiology* **47**: 133-140.

Baumann L, Doughty T, Siewers V, Nielsen J, Boles E & Oreb M (2021) Transcriptomic response of *Saccharomyces cerevisiae* to octanoic acid production. *FEMS Yeast Research* **21**.

Bennis NX, Kostanjšek M, van den Broek M & Daran JG (2023) Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts. *New Biotechnology* **76**: 49-62.

Besada-Lombana PB, Fernandez-Moya R, Fenster J & Da Silva NA (2017) Engineering *Saccharomyces cerevisiae* fatty acid composition for increased tolerance to octanoic acid. *Biotechnology and Bioengineering* **114**: 1531-1538.

Brickwedde A, van den Broek M, Geertman JA, Magalhães F, Kuijpers NGA, Gibson B, Pronk JT & Daran JG (2017) Evolutionary engineering in chemostat cultures for improved maltotriose fermentation kinetics in *Saccharomyces pastorianus* lager brewing yeast. *Frontiers in Microbiology* **8**: 1690.

Clapperton JF (1978) Fatty acids contributing to caprylic flavour in beer. The use of profile and threshold data in flavour research. *Journal of the Institute of Brewing* **84**: 107-112.

Dunn B & Sherlock G (2008) Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. *Genome research* **18**: 1610-1623.

Enari T-M (1977) E.B.C. Analytica microbiologica. *Journal of the Institute of Brewing* **83**: 109-118.

Engan S (1972) Organoleptic threshold values of some alcohols and esters in beer. *Journal of the Institute of Brewing* **78**: 33-36.

Entian K-D & Kötter P (2007) 25 yeast genetic strain and plasmid collections. *Methods in microbiology* **36**: 629-666.

Fernandez-Moya R & Da Silva NA (2017) Engineering *Saccharomyces cerevisiae* for high-level synthesis of fatty acids and derived products. *FEMS Yeast Research* **17**.

Ferreira IM & Guido LF (2018) Impact of wort amino acids on beer flavour: A Review. *Fermentation* **4**: 23.

Fujii T, Nagasawa N, Iwamatsu A, Bogaki T, Tamai Y & Hamachi M (1994) Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. *Applied and Environmental Microbiology* **60**: 2786-2792.

Fujii T, Yoshimoto H, Nagasawa N, Bogaki T, Tamai Y & Hamachi M (1996) Nucleotide sequences of alcohol acetyltransferase genes from lager brewing yeast, *Saccharomyces carlsbergensis*. *Yeast* **12**: 593-598.

Furukawa K, Yamada T, Mizoguchi H & Hara S (2003) Increased ethyl caproate production by inositol limitation in *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering* **95**: 448-454.

Gajewski J, Pavlovic R, Fischer M, Boles E & Grininger M (2017b) Engineering fungal *de novo* fatty acid synthesis for short chain fatty acid production. *Nature Communications* **8**: 14650.

Gajewski J, Buelens F, Serdjukow S, Janßen M, Cortina N, Grubmüller H & Grininger M (2017a) Engineering fatty acid synthases for directed polyketide production. *Nature Chemical Biology* **13**: 363-365.

Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA & Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* **6**: 343-345.

Gorter de Vries AR, de Groot PA, van den Broek M & Daran J-MG (2017) CRISPR-Cas9 mediated gene deletions in lager yeast *Saccharomyces pastorianus*. *Microbial Cell Factories* **16**: 222.

Gorter de Vries AR, Knibbe E, van Roosmalen R, *et al.* (2020) Improving industrially relevant phenotypic traits by engineering chromosome copy number in *Saccharomyces pastorianus*. *Frontiers in Genetics* 11: 518.

Hassing E-J, de Groot PA, Marquenie VR, Pronk JT & Daran J-MG (2019) Connecting central carbon and aromatic amino acid metabolisms to improve *de novo* 2-phenylethanol production in *Saccharomyces cerevisiae*. *Metabolic Engineering* **56**: 165-180.

Hazelwood LA, Daran JM, van Maris AJ, Pronk JT & Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Applied Environmental Microbiology* **74**: 2259-2266.

Hundová Z & Fencl Z (1977) Toxic effects of fatty acids on yeast cells: Dependence of inhibitory effects on fatty acid concentration. *Biotechnology and Bioengineering* **19**: 1623-1641.

Knight MJ, Bull ID & Curnow P (2014) The yeast enzyme Eht1 is an octanoyl-CoA:ethanol acyltransferase that also functions as a thioesterase. *Yeast* **31**: 463-474.

Leber C, Polson B, Fernandez-Moya R & Da Silva NA (2015) Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in

Saccharomyces cerevisiae. *Metabolic Engineering* **28**: 54-62.

Lee ME, DeLoache WC, Cervantes B & Dueber JE (2015) A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synthetic Biology* **4**: 975-986.

Legras JL, Erny C, Le Jeune C, Lollier M, Adolphe Y, Demuyter C, Delobel P, Blondin B & Karst F (2010) Activation of two different resistance mechanisms in *Saccharomyces cerevisiae* upon exposure to octanoic and decanoic acids. *Applied Environmental Microbiology* **76**: 7526-7535.

Li H (2018) Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* **34**: 3094-3100.

Li H & Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754-1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G & Durbin R (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**: 2078-2079.

Libkind D, Hittinger CT, Valério E, Gonçalves C, Dover J, Johnston M, Gonçalves P & Sampaio JP (2011) Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences* **108**: 14539-14544.

Liu P-T, Zhang B-Q, Duan C-Q & Yan G-L (2020) Pre-fermentative supplementation of unsaturated fatty acids alters the effect of overexpressing *ATF1* and *EEB1* on esters biosynthesis in red wine. *Lwt* **120**.

Liu P, Chernyshov A, Najdi T, Fu Y, Dickerson J, Sandmeyer S & Jarboe L (2013) Membrane stress caused by octanoic acid in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **97**: 3239-3251.

Lõoke M, Kristjuhan K & Kristjuhan A (2011) Extraction of genomic DNA from yeasts for PCR-based applications. *Biotechniques* **50**: 325-328.

Mason AB & Dufour JP (2000) Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* **16**: 1287-1298.

Meilgaard MC (1982) Prediction of flavor differences between beers from their chemical composition. *Journal of Agricultural and Food Chemistry* **30**: 1009-1017.

Nakao Y, Kanamori T, Itoh T, Kodama Y, Rainieri S, Nakamura N, Shimonaga T, Hattori M & Ashikari T (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Research* **16**: 115-129.

Nijkamp JF, van den Broek MA, Geertman J-MA, Reinders MJT, Daran J-MG & de Ridder D (2012) *De novo* detection of copy number variation by co-assembly. *Bioinformatics* **28**: 3195-3202.

Nordström K (1964) Formation of esters from acids by brewer's yeast. IV. Effect of higher fatty acids and toxicity of lower fatty acids. *Journal of the Institute of Brewing* **70**: 233–238.

Nykänen L, Nykänen I & Suomalainen H (1977) Distribution of esters produced during supar fermentation between the yeast cell and the medium. *Journal of the Institute of Brewing* **83**: 32-34.

Olaniran AO, Hiralal L, Mokoena MP & Pillay B (2017) Flavour-active volatile compounds in beer: production, regulation and control. *Journal of the Institute of Brewing* **123**: 13-23.

Peddie HAB (1990) Ester formation in brewery fermentations. *Journal of the Institute of Brewing* **96**: 327-331.

Pires EJ, Teixeira JA, Brányik T & Vicente AA (2014) Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology* **98**: 1937-1949.

Renger RS, Hateren SHv & Luyben KCAM (1992) The formation of esters and higher alcohols during brewery fermentation: The effect of carbon dioxide pressure. *Journal of the Institute of Brewing* **98**: 509-513.

Romagnoli G, Luttik MA, Kötter P, Pronk JT & Daran JM (2012) Substrate specificity of thiamine pyrophosphate-dependent 2-oxo-acid decarboxylases in *Saccharomyces cerevisiae*. *Applied Environmental Microbiology* **78**: 7538-7548.

Saerens SM, Delvaux FR, Verstrepen KJ & Thevelein JM (2010) Production and biological function of volatile esters in *Saccharomyces cerevisiae*. *Microbial Biotechnology* **3**: 165-177.

Saerens SM, Delvaux F, Verstrepen KJ, Van Dijck P, Thevelein JM & Delvaux FR (2008) Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Applied and Environmental Microbiology* **74**: 454-461.

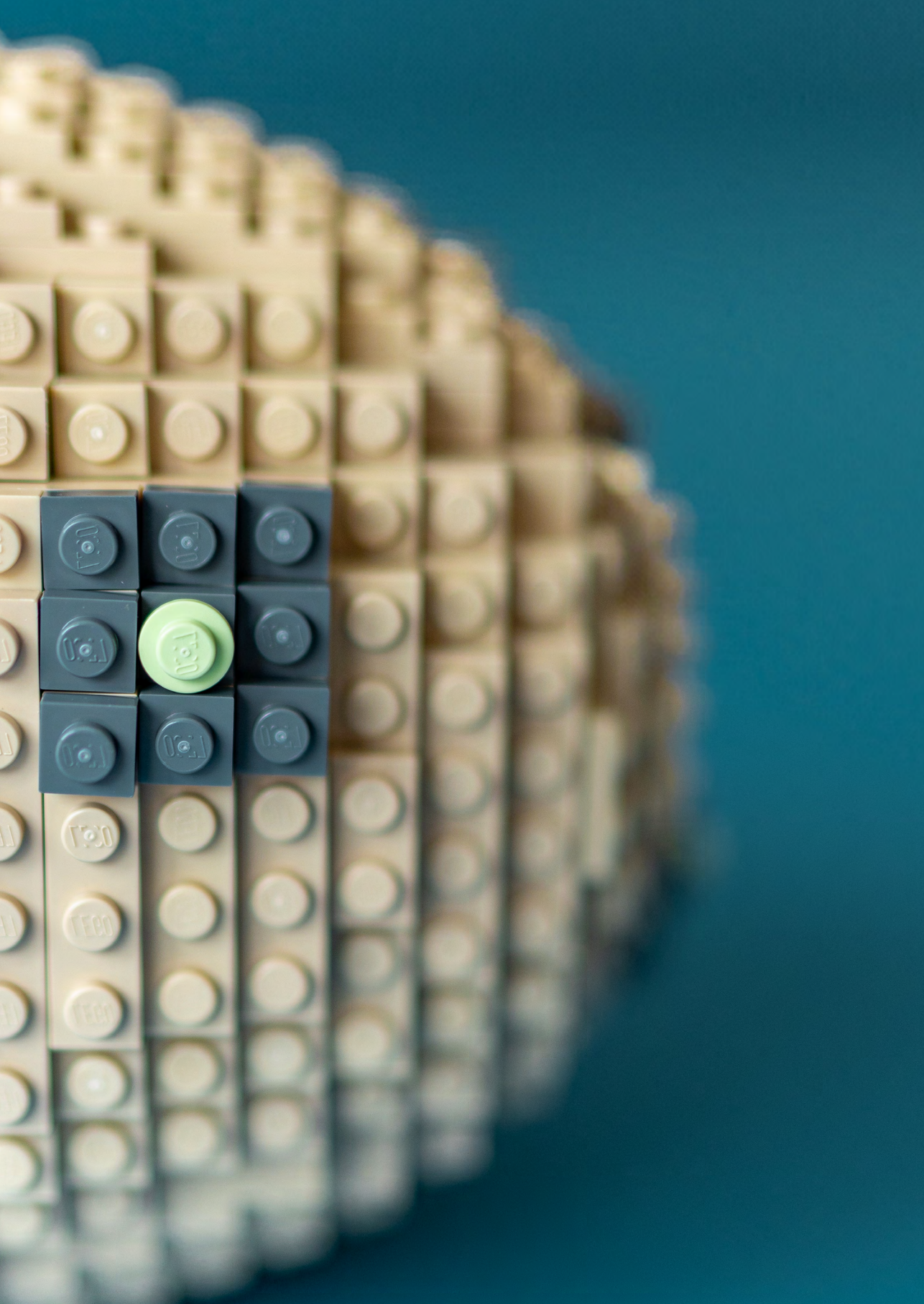
Saerens SM, Verstrepen KJ, Van Laere SD, Voet AR, Van Dijck P, Delvaux FR & Thevelein JM (2006) The *Saccharomyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. *Journal of Biological Chemistry* **281**: 4446-4456.

Salazar AN, Gorter de Vries AR, van den Broek M, Brouwers N, de la Torre Cortès P, Kuijpers NGA, Daran JG & Abeel T (2019) Chromosome level assembly and comparative genome analysis confirm lager-brewing yeasts originated from a single hybridization. *BMC Genomics* **20**: 916.

Schieberle P & Hofmann T (1997) Evaluation of the character impact odorants in fresh strawberry juice by quantitative measurements and sensory studies on model mixtures. *Journal of Agricultural and Food Chemistry* **45**: 227-232.

Solis-Escalante D, Kuijpers NG, Bongaerts N, Bolat I, Bosman L, Pronk JT, Daran JM & Daran-Lapujade P

(2013) amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. *FEMS Yeast Research* **13**: 126-139.
Stewart GG (2017) Flavour production by yeast. *Brewing and Distilling Yeasts*, 325-355.
Suomalainen H (1981) Yeast esterases and aroma esters in alcoholic beverages. *Journal of the Institute of Brewing* **87**: 296-300.
Thorvaldsdóttir H, Robinson JT & Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in Bioinformatics* **14**: 178-192.
Verstrepen KJ, Derdelinckx G, Dufour J-P, Winderickx J, Thevelein JM, Pretorius IS & Delvaux FR (2003a) Flavor-active esters: Adding fruitiness to beer. *Journal of Bioscience and Bioengineering* **96**: 110-118.
Verstrepen KJ, Van Laere SD, Vanderhaegen BM, Derdelinckx G, Dufour JP, Pretorius IS, Winderickx J, Thevelein JM & Delvaux FR (2003b) Expression levels of the yeast alcohol acetyltransferase genes *ATF1*, *Lg-ATF1*, and *ATF2* control the formation of a broad range of volatile esters. *Applied and Environmental Microbiology* **69**: 5228-5237.
Wakil SJ, Stoops JK & Joshi VC (1983) Fatty acid synthesis and its regulation. *Annual Review of Biochemistry* **52**: 537-579.
Wernig F, Born S, Boles E, Grininger M & Oreb M (2020) Fusing α and β subunits of the fungal fatty acid synthase leads to improved production of fatty acids. *Scientific Reports* **10**: 9780.
Xi Z-m, Tao Y-s, Zhang L & Li H (2011) Impact of cover crops in vineyard on the aroma compounds of *Vitis vinifera* L. *cv* Cabernet Sauvignon wine. *Food Chemistry* **127**: 516-522.
Yin H, Liu L-P, Yang M, Ding X-T, Jia S-R, Dong J-J & Zhong C (2019) Enhancing medium-chain fatty acid ethyl ester production during beer fermentation through *EEB1* and *ETR1* overexpression in *Saccharomyces pastorianus*. *Journal of Agricultural and Food Chemistry* **67**: 5607-5613.
Yoshimoto H, Fujiwara D, Momma T, Ito C, Sone H, Kaneko Y & Tamai Y (1998) Characterization of the *ATF1* and *Lg-ATF1* genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*. *Journal of Fermentation and Bioengineering* **86**: 15-20.
Yoshimoto H, Fujiwara D, Momma T, Tanaka K, Sone H, Nagasawa N & Tamai Y (1999) Isolation and characterization of the *ATF2* gene encoding alcohol acetyltransferase II in the bottom fermenting yeast *Saccharomyces pastorianus*. *Yeast* **15**: 409-417.



Chapter 6

Outlook and valorisation aspects

6.1. Outlook and valorisation aspects

The fast and transformative developments in genome editing, sequencing technologies and data science have accelerated fundamental and application-inspired research in the life sciences, with important current and future impacts on society (Netherlands Commission on Genetic Modification (COGEM), 2023). These developments have also contributed to making the budding yeast *Saccharomyces cerevisiae* the genetically and physiologically best characterized and genetically most accessible eukaryote. As a result, *S. cerevisiae* is an intensively used model for research on healthy and diseased human cells and a 'jack of all trades' workhorse in microbial biotechnology, with products ranging from car fuel to pharmaceuticals.

S. cerevisiae × *S. eubayanus* hybrids known as *S. pastorianus* are of immense economic importance due to their role in brewing of lager-type beers. Major breakthroughs regarding the understanding and engineering of these yeasts are, however, impeded by the complexity of their hybrid genomes, limited knowledge on their metabolic network and its regulation, and an incomplete understanding on how alleles derived from the two parental species contribute to shaping their hybrid phenotypes.

Next-generation short-read sequencing technologies, provided by companies such as Illumina and Ion Torrent, and third-generation long-read sequencing platforms, provided by companies such as Oxford Nanopore Technology and Pacific Biosciences, contributed to sequencing with much higher throughput, increased accuracy, rapid pace and diverse outputs (Hyman, 1988, Schadt *et al.*, 2010). Real-time single molecule DNA sequencing has been integrated in standardized laboratory procedures, while RNA or even protein sequencing and single-cell genome sequencing are progressing towards a similar level of accessibility (McCarthy, 2010, Alfaro *et al.*, 2021, Hu *et al.*, 2021, Wang *et al.*, 2021). Combining long (10 kbp to 100 kbp) and short (50 to 300 bp) sequencing reads allows for highly complete and accurate chromosome-level genome assemblies. In particular, the use of long reads enables the generation of much less fragmented genome assemblies and can also capture sequences that contain repeats. This advantage contributes to improved resolution of the sequences of (sub)telomeric regions, which harbour industrially relevant sequences, such as *MAL* loci and *FLO* genes. Complete, high-quality genome assemblies are essential to investigate the contribution of such genes to brewing-related phenotypes. High-coverage short-read sequencing remains a highly valuable and complementary tool for determining copy numbers of chromosomes and/or individual alleles and allows for accurate heterozygosity mapping.

Assembly of complete and accurate genome sequences of diploid, polyploid or alloploid yeasts remains challenging. Currently, two fully assembled and annotated genomes of two *S. pastorianus* strains are available: WS34/70 (Nakao *et al.*, 2009) and CBS 1483 (Salazar *et al.*, 2019). These chromosome-level genomes of *S. pastorianus*

contribute to a comprehensive understanding of genotype to phenotype relations, genome plasticity and evolutionary history. Furthermore, these currently available and new reference genomes are essential for efficient genome engineering in the context of fundamental research and industrial strain improvement. However, the haploid representation of these genomes offers only snapshots of reality (Patterson *et al.*, 2015, Abou Saada *et al.*, 2021, Shirali Hossein Zade *et al.*, 2022). Chromosome-level assemblies that provide a single sequence per chromosome yield a consensus assembly that fails to capture chromosome heterozygosity, which can be a significant source of genetic variation. The ability to reconstruct complete chromosomes including copy-specific allelic variations would enable analysis of heterozygosity, structural variants and allele-specific phenotypes. Such haplotyping has been performed on diploid genomes, including the human genome (Wenger *et al.*, 2019). However, applying haplotyping to allo-aneuploid genomes, including those of brewing yeasts, remains challenging due to the polyploidy and copy number differences between chromosomes. Haplotype-level genome assemblies of brewing yeast generated from raw sequencing data will contribute to understanding contributions of allelic variants to phenotypes, structural variation, history of hybridization, and will improve the efficiency of genetic engineering. To allow for implementation of this approach in brewing yeast research, DNA-sequencing platforms capable of generating longer reads with (even) lower error rates are required, as well as user-friendly algorithms for analysing the resulting sequencing outputs.

The CRISPR-Cas DNA-editing technology has transformed genetic engineering and modification of *S. cerevisiae*. Innovations in CRISPR-Cas editing methodologies have been validated for rapid, highly accurate and multiplexed genome editing. The gEL DNA methodology described in **Chapter 2** has eliminated the laborious PCR amplification and cloning steps, thereby allowing high-throughput *S. cerevisiae* strain construction in shorter time frames. The continuous search for new, alternative CRISPR endonucleases with greater flexibility in design options, for example by their use of different PAM recognition sequences, improved targeting efficiencies (Hu *et al.*, 2018), alternative crRNA expression systems and multiplex editing possibilities, will continue to further expand the genetic toolbox for genetic modification of *S. cerevisiae*. Furthermore, other CRISPR-based technologies that rely on inactive Cas protein (dead Cas) (Jinek *et al.*, 2012, Qi *et al.*, 2013), nicking Cas protein (nicking Cas) (Cong *et al.*, 2013) and fusion proteins consisting of Cas variants combined with deaminases (base editors) (Komor *et al.*, 2016, Nishida *et al.*, 2016), reverse transcriptases (prime editors) (Anzalone *et al.*, 2019), transcriptional effectors (Bikard *et al.*, 2013, Perez-Pinera *et al.*, 2013), or engineered DNA polymerases for targeted mutagenesis (Halperin *et al.*, 2018) have been developed, each with their tailored application. Cas proteins active on RNA (East-Seletsky *et al.*, 2017) or proteins (Hu *et al.*, 2022) even further expand the molecular toolbox. Combination of orthogonal CRISPR-Cas systems are bound to unlock even more sophisticated applications in the future.

One of the primary challenges associated with CRISPR-Cas systems is the species-specific variation in their editing efficiency, coupled with the imperfect predictability of the editing efficiency for individual gRNA sequences. Applicability of CRISPR-Cas architectures has been extended based on gRNA-expression cassettes relying on RNA polymerase II or III, carried on different expression vectors, and by using Cas expression systems optimized for target species. However, application of these systems to non-conventional yeast species is still challenging due to unknown intrinsic cellular processes affecting CRISPR-editing. Until context-dependency of gRNA efficiency is further elucidated, simultaneous testing of multiple gRNA designs may be required for fast and efficient editing of new and more common hosts.

The complex patent landscape for Cas-based genome editing, especially for the hallmark endonucleases *Spy*Cas9 (Jinek *et al.*, 2012) and *Fn*Cas12a (Zetsche *et al.*, 2015), poses a hurdle for applying the technology to industrial strains. This also holds true when the commercial end product no longer contains genetic information that can be traced to the applied Cas nuclease or gRNA. Access to CRISPR systems with clearer and less restrictive IP rights can promote democratization of the CRISPR editing technologies. Next to having interesting editing characteristics, *Er*Cas12a is currently the only Cas nuclease with a free commercial research license (**Chapter 3**). While the patent 'war' on CRISPR-Cas technologies continues (Ledford, 2017, Ledford, 2022), it is interesting to note that the first patents, filed in 2013, will expire in about a decade, thereby clearing the room for royalty-free application of this world-changing technology.

While CRISPR-Cas genome editing in haploid yeast species is relatively straightforward, editing of the genomes of diploid, or more generally, polyploid heterozygous yeasts is drastically impaired when not all chromosomes carrying a (homoeolog) of the targeted allele can be targeted by the same gRNA (Gorter de Vries *et al.*, 2018) (**Chapter 4**). In such scenarios, massive and unpredictable loss of heterozygosity, extending far beyond the targeted locus, can result from homology-directed repair with an intact homologous chromosome, instead of the provided repair fragment, as repair template. This loss of heterozygosity mechanism predominates the outcome of CRISPR-Cas9 genome editing in heterozygous diploid and hybrid (alloploid) yeast, such as *S. pastorianus*, and *Zygosaccharomyces parabaiili* (Gorter de Vries *et al.*, 2018, Bennis *et al.*, 2023, Jayaprakash *et al.*, 2023), thereby hindering gene editing of these hybrid yeasts. **Chapter 4** demonstrates that genome regions in *S. pastorianus* strains that are devoid of homoeologous regions can be used as 'safe' targets for CRISPR-Cas9-mediated integration of expression cassettes. The ability to efficiently integrate genes in *S. pastorianus* and potentially other hybrid yeasts provides new opportunities to accurately and time-effectively engineer strains with new characteristics. However, gene deletions and subtle sequence modifications at specific loci requires other measures. Simultaneous targeting of all chromosome homoeologs avoids loss of

heterozygosity and enables modifications of the gene of interest. However, studying allele-specific contributions to phenotypes using allele specific knock-out strains or gene-specific homozyogte strains is severely impaired under this condition, as generation of these strains require allele-specific editing (Gorter de Vries *et al.*, 2017). Alternative genetic engineering tools that do not result in loss of heterozygosity upon targeting heterozygote sequences remain to be developed for *S. pastorianus* and other hybrid yeasts to achieve these types of genetic modifications.

Genetic engineering has the potential to swiftly, safely and efficiently improve a wide range of phenotypic traits and fermentation characteristics of brewing yeasts, including wort sugar utilization, fermentation rate and energetic performance, reduction of off-flavours (Gjermansen *et al.*, 1988, Sone *et al.*, 1988, Fujii *et al.*, 1990, Blomqvist *et al.*, 1991, Yamano *et al.*, 1994, Yamano *et al.*, 1995, Guo *et al.*, 2001) (**Chapter 4**) and balanced flavour profiles (Yin *et al.*, 2019) (**Chapter 5**), and implementation of novel flavours (Denby *et al.*, 2018). In contrast to metabolic engineering efforts for production of chemicals, which aim to maximize titer, rate and yield ('TRY'), targets in strain improvement of brewing yeasts are much more subtle. Flavour palette, taste and other sensory qualities of beer requires fine-tuning of the metabolic fluxes towards multiple desired and less-desired compounds (Denby *et al.*, 2018). Ideally, a plug-and-play brewing yeast toolkit containing thoroughly characterized native gene overexpression cassettes, gene deletion fragments, gene mutants and heterologous genes would be available to allow editing of different brewing yeast strains. An outline of such a toolkit is already emerging with strategies for introducing acetolactate decarboxylases (eliminating diacetyl formation), sugar transporters (extending or improving substrate uptake capacity), monoterpene synthases (production of hop compounds) and ethyl ester biosynthesis genes (tuning and extending aroma profiles). This development is likely to continue to expand with future metabolic engineering efforts in *S. pastorianus* allowing tailored, on-demand strain design, construction and implementation.

The growing demand for diversification of beers is reflected in the strong upsurge of craft- and microbreweries, which prioritize the production of distinctive and innovative beers, emphasising quality and flavour uniqueness. While craft breweries are rapidly entering the booming market, the large beer companies remain rather conservative. The use of genetically modified yeasts is a potential risk for market sales of established companies, resulting from society's view on acceptance on genetic modification. On the other hand, craft and micro-breweries aim at these more progressive beer markets. Especially, brewing yeasts engineered for unique flavours palettes or more environmentally sustainable beer production are attractive for explorative, health-aware or environmental-conscious consumers. The latter includes eliminating the energy intensive lagering step, replacing hops (cultivation emission of 4.0 kg CO_2 kg^{-1}) by yeast-derived hop flavours, or more efficient use of sugars for process optimalisation

and intensification. Yeast producers, including Berkeley Yeast, Lallemand Brewing and Omega Yeast, all located in the US, are rapidly developing and commercializing such genetically engineered yeast strains. It will be very interesting to see if entrepreneurs with the drive and stamina needed to navigate the European regulatory and public-acceptance landscape will introduce this trend in Europe as well.

Especially in Europe, acceptance of genetic engineering technologies by society remains the biggest challenge for large-scale market introduction of beer brewed with engineered yeasts. Addressing and resolving societal concerns regarding the use of genetically modified microorganisms in the food and beverage industry is essential to be able to realize their potential commercial and societal value. Applying genetic engineering techniques to increase the sustainability of a food or beverage production process, to produce healthier (e.g. low calorie) products, to intensify production processes or diversifying the products, could contribute to a more positive societal attitude towards these innovative techniques. Public awareness of the similarities and differences of classical strain improvement approaches, which have been applied at a large scale for the better part of a century, and targeted strain engineering techniques, which despite the half-century history of recombinant-DNA technology are still concerned by many as ultra-new, is very low. Raising understanding of the potential benefits of modern biotechnology, while openly and honestly discussing emotions and (perceived) risks related to its application in the food and beverage industry continues to be important.

Rapid developments in precision fermentation might stimulate the acceptance of genetically modified microorganisms for food and beverage production. Precision fermentation relies on the use of specifically engineered microorganisms as cell factories to produce high-value functional food ingredients with high yield and purity. One promising example is single-cell protein (SCP) production in which microorganisms convert renewable 'zero-emission' feedstocks to protein-rich biomass as protein complement or substitute for food and feed supply by the agricultural industry. As the world population will outgrow the nutritional resources supplied by agriculture, SCP production is a promising technology to tackle the protein deficiency problem worldwide. Increasing the protein yield, changing biomass composition to increase its nutritional value, altering the starting material or improving the production process are potential (genetic) engineering targets. The path towards acceptance in the food and beverage industry is still under construction and will, also in the coming years, require patience and persistence from, amongst others, scientists.

6.2. References

Abou Saada O, Tsouris A, Eberlein C, Friedrich A & Schacherer J (2021) nPhase: an accurate and contiguous phasing method for polyploids. *Genome Biology* **22**: 126.

Alfaro JA, Bohländer P, Dai M, *et al.* (2021) The emerging landscape of single-molecule protein sequencing technologies. *Nature Methods* **18**: 604-617.

Anzalone AV, Randolph PB, Davis JR, et al. (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576: 149-157.

Bennis NX, Kostanjšek M, van den Broek M & Daran JG (2023) Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts. *New Biotechnology* **76**: 49-62.

Bikard D, Jiang W, Samai P, Hochschild A, Zhang F & Marraffini LA (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Research* **41**: 7429-7437.

Blomqvist K, Suihko M-L, Knowles J & Penttilä M (1991) Chromosomal integration and expression of two bacterial α-acetolactate decarboxylase genes in brewer's yeast. *Applied and Environmental Microbiology* **57**: 2796-2803.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**: 819-823.

Denby CM, Li RA, Vu VT, Costello Z, Lin W, Chan LJG, Williams J, Donaldson B, Bamforth CW, Petzold CJ, Scheller HV, Martin HG, Keasling JD (2018) Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer. *Nature Communications* **9**: 965.

East-Seletsky A, O'Connell MR, Burstein D, Knott GJ & Doudna JA (2017) RNA targeting by functionally orthogonal type VI-A CRISPR-Cas enzymes. *Molecular Cell* **66**: 373-383.

Fujii T, Kondo K, Shimizu F, Sone H, Tanaka J & Inoue T (1990) Application of a ribosomal DNA integration vector in the construction of a brewer's yeast having α-acetolactate decarboxylase activity. *Applied and Environmental Microbiology* **56**: 997-1003.

Gjermansen C, Nilsson-Tillgren T, Petersen JGL, Kielland-Brandt MC, Sigsgaard P & Holmberg S (1988) Towards diacetyl-less brewers' yeast. Influence of ilv2 and ilv5 mutations. *Journal of Basic Microbiology* **28**: 175-183.

Gorter de Vries AR, de Groot PA, van den Broek M & Daran J-MG (2017) CRISPR-Cas9 mediated gene deletions in lager yeast *Saccharomyces pastorianus*. *Microbial Cell Factories* **16**: 222.

Gorter de Vries AR, Couwenberg LGF, van den Broek M, de la Torre Cortés P, ter Horst J, Pronk JT & Daran J-MG (2018) Allele-specific genome editing using CRISPR–Cas9 is associated with loss of heterozygosity in diploid yeast. *Nucleic Acids Research* **47**: 1362-1372.

Guo W, He X, Tie C & Zhang B (2001) Expression of α-acetolactate decarboxylase gene from *Bacillus subtilis* in brewer's yeast. *Wei Sheng Wu Xue Bao* **41**: 105-108.

Halperin SO, Tou CJ, Wong EB, Modavi C, Schaffer DV & Dueber JE (2018) CRISPR-guided DNA polymerases enable diversification of all nucleotides in a tunable window. *Nature* **560**: 248-252.

Hu C, van Beljouw SPB, Nam KH, Schuler G, Ding F, Cui Y, Rodríguez-Molina A, Haagsma AC, Valk M, Pabst M, Brouns SJJ, Ke A (2022) Craspase is a CRISPR RNA-guided, RNA-activated protease. *Science* **377**: 1278-1285.

Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* **556**: 57-63.

Hu T, Chitnis N, Monos D & Dinh A (2021) Next-generation sequencing technologies: An overview. *Human Immunology* **82**: 801-811.

Hyman ED (1988) A new method of sequencing DNA. *Analytical Biochemistry* **174**: 423-436.

Jayaprakash P, Barroso L, Vajente M, Maestroni L, Louis EJ, Morrissey JP & Branduardi P (2023) CRISPR-Cas9 engineering in the hybrid yeast *Zygosaccharomyces parabailii* can lead to loss of heterozygosity in target chromosomes. *FEMS Yeast Research* **23**.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA & Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816-821.

Komor AC, Kim YB, Packer MS, Zuris JA & Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**: 420-424.

Ledford H (2017) Bitter CRISPR patent war intensifies. Nature.

Ledford H (2022) Major CRISPR patent decision won't end tangled dispute. *Nature* **603**: 373-374.

McCarthy A (2010) Third generation DNA sequencing: pacific biosciences' single molecule real time technology. *Chemistry & Biology* **17**: 675-676.

Nakao Y, Kanamori T, Itoh T, Kodama Y, Rainieri S, Nakamura N, Shimonaga T, Hattori M & Ashikari T (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Research* **16**: 115-129.

Netherlands Commission on Genetic Modification (COGEM) (2023) Biotechnology trend analysis 2023, A call for vision, decision and direction. *COGEM*.

Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M,

Hara KY, Shimatani Z, Kondo A (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* **353**.

Patterson M, Marschall T, Pisanti N, van Iersel L, Stougie L, Klau GW & Schönhuth A (2015) WhatsHap: Weighted Haplotype Assembly for Future-Generation Sequencing Reads. *Journal of Computational Biology* **22**: 498-509.

Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, Thakore PI, Glass KA, Ousterout DG, Leong KW, Guilak F, Crawford GE, Reddy TE, Gersbach CA (2013) RNA-guided gene activation by CRISPR-Cas9–based transcription factors. *Nature Methods* **10**: 973-976.

Qi Lei S, Larson Matthew H, Gilbert Luke A, Doudna Jennifer A, Weissman Jonathan S, Arkin Adam P & Lim Wendell A (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**: 1173-1183.

Salazar AN, Gorter de Vries AR, van den Broek M, Brouwers N, de la Torre Cortès P, Kuijpers NGA, Daran J-MG & Abeel T (2019) Chromosome level assembly and comparative genome analysis confirm lager-brewing yeasts originated from a single hybridization. *BMC Genomics* **20**: 916.

Schadt EE, Turner S & Kasarskis A (2010) A window into third-generation sequencing. *Human Molecular Genetics* **19**: R227-R240.

Shirali Hossein Zade R, Urhan A, Assis de Souza A, Singh A & Abeel T (2022) HAT: haplotype assembly tool using short and error-prone long reads. *Bioinformatics* **38**: 5352-5359.

Sone H, Fujii T, Kondo K, Shimizu F, Tanaka J & Inoue T (1988) Nucleotide sequence and expression of the *Enterobacter aerogenes* α-acetolactate decarboxylase gene in brewer's yeast. *Applied and Environmental Microbiology* **54**: 38-42.

Wang Y, Zhao Y, Bollas A, Wang Y & Au KF (2021) Nanopore sequencing technology, bioinformatics and applications. *Nature Biotechnology* **39**: 1348-1365.

Wenger AM, Peluso P, Rowell WJ, *et al.* (2019) Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome. *Nature Biotechnology* **37**: 1155-1162.

Yamano S, Tanaka J & Inoue T (1994) Cloning and expression of the gene encoding α-acetolactate decarboxylase from *Acetobacter aceti ssp. xylinum* in brewer's yeast. *Journal of Biotechnology* **32**: 165-171.

Yamano S, Tomizuka K, Sone H, Imura M, Takeuchi t, Tanaka J & Inoue T (1995) Brewing performance of a brewer's yeast having α-acetolactate decarboxylase from *Acetobacter aceti subsp. xylinum*. *Journal of Biotechnology* **39**: 21-26.

Yin H, Liu L-P, Yang M, Ding X-T, Jia S-R, Dong J-J & Zhong C (2019) Enhancing medium-chain fatty acid ethyl ester production during beer fermentation through *EEB1* and *ETR1* overexpression in *Saccharomyces pastorianus*. *Journal of Agricultural and Food Chemistry* **67**: 5607-5613.

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, Koonin EV, Zhang F (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**: 759-771.

Acknowledgements

Embarking on a PhD trajectory is stepping into a labyrinth where each twist and turn presents new challenges, insights and revelations. The path to a doctoral degree is intertwined with endless support, unwavering encouragement, immeasurable inspiration and collective wisdom of mentors, colleagues, friends and family. The past four years have been truly a wonderful experience and I am deeply grateful to everyone that played a role in this journey.

First and foremost, my gratitude goes to my promoter and supervisor, **Jean-Marc**, for the support and guidance during the project. Your seemingly limitless knowledge on yeasts, physiology, brewing, molecular biology and genomics is truly astonishing and very valuable. I appreciate the scientific advice, freedom and trust that you gave me, which allowed to develop myself into the researcher I am today. It is a pleasure working with you and I am looking forward to the upcoming challenges in the next project as postdoc.

Many thanks to **Jack** for being my second promoter. Your enthusiasm and passion for biotechnology is rather contagious, as my interest for biotechnology was actually boosted by the bachelor course lectures presented by Ton and you. Being infected with the 'biotech-virus' is actually a nice one to catch. I really enjoyed the path that I took which was partly chosen based on your lectures. Teaching this particular course together with you and the other assistants has been great fun! Although your involvement in my PhD has been restricted due to your role as head of the department, I appreciate our discussions on science, personal developments and career paths, as I always left your office or the lunch walk, or hung up the phone, with valuable advice.

Pascale, although you were not directly involved in my PhD project, I have learned a lot of valuable skills from you. Going back in time, my bachelor and master thesis under your supervision laid the foundation for the skills proven essential throughout my PhD. Being part of your Metabolic Reprogramming teaching team was a great experience and a lot of fun (especially when correcting the exams together). Our collaboration on the 'gEL DNA' methodology resulted in a wonderful research publication. Finally, we went on two conference trips together (ASBE VI in Edinburgh, 2022 and ICY16 in Cape Town, 2024) and both resulted in fun memories.

The IMB research group is rich in PI's, each with a different expertise, knowledge and methods. **Robert**, **Walter**, **Rinke**, **Djordje** and **Marcel VL**, thanks you for all your contributions to the research group. I wish you all the luck in the future!

One of the most important resources for PhD candidates of the research group IMB are technicians. **Erik**, **Marcel vdB**, **Marijke**, **Clara**, **Christiaan**, **Agostina**, **Pilar** and **Jordi**, each of you is (or has been) one of the cornerstones of all experimental work conducted at IMB. Your expertise in fermentations, bioinformatics, sequencing, molecular biology, enzyme kinetics, laboratory set-ups, neurolinguistic programming and more, is worth its weight in gold. Most of us PhDs candidates, including myself, would probably be

lost and running around like headless chickens without you.

Special thanks to **Marcel** for being a bioinformatic mastermind and very kind colleague. We have spent an ernormous amount of time together in your office identifying the landing sites of CBS 1483, solving the CBS 1513 genome assembly puzzle, figuring out how to deal with transcriptomics data and many more 'straightforward' spin-off projects that always turned out to be more complicated than anticipated. At the start of my PhD, I would never have thought that I would be able to assemble genomes. You are a great listener and very patient teacher and I really enjoyed learning from you and working together with you on all the bioinformatic parts of the projects that are part of this thesis, but also on all the other projects.

Erik, throughout my whole PhD I never ever touched your bioreactors… Nonetheless, we spent quite some time together in- and outside the lab. You are always there to help out in the lab: when a water bath decided to burn up, when a sampling port suddenly broke down, the power supply shut down, or when shaker spots were in desperate need. You are always up for initiating and joining the diverse social activities that mostly went hand-in-hand with ordering a couple of beers. The international conference trips to ASBE VI in Edinburgh and PYFF8 in Cork were also lots of fun and resulted in great memories! I hope that we stay friends and have many more bike rides Delft-Pijnacker after the Friday drinks together.

Marijke, you are an inspiring person with a heart of gold. I appreciate your patience and willingness to always help out, especially in challenging moments. Your ability to let others shine is very admirable. Thank you for all the support with regards to science, but equally important, your guidance and stimulation to grow as a person. Finally, the trip to Cape Town with you is one that I will remember!

Working with microorganisms at IMB requires media, specific chemicals, vast amounts of clean glassware and huge quantities of laboratory supplies. **Apilena**, **Jannie** and **Gea** (and **Astrid**) (also known as 'the MSD ladies') thank you for running endless autoclave programs with my tall tubes, the uncountable number of media bottles that I used, the tremendous amounts of shake flasks that you have prepared, and for always being prepared for experiments that got a bit out of hand. The fact that you always stepped in when needed has often saved my experimental planning. Something I also very much enjoyed were our regular chats in the lab. I wish you all the best.

I had the privilege to work closely with the HTS-team. **Jasper**, **Susan** and **Marìa**, you were essential at the start of my PhD project when I barely knew anything about brewing and brewing yeasts. **Susan**, you might not even remember it, but I really appreciated your outreach to me when I started my PhD home alone and 'all by myself' due to COVID19 times. **Marìa**, during the time we shared the 'premium'-office on the first floor, I got to know you very well. Your well-established brewing-related

experimental protocols were highly valuable for some chapters in this thesis. Although you live in Ghent now, I hope our friendship will continue. Thank you for all the walks, chats and fun! **Ewout**, **Flip** and **Ilja**, when you joined the HTS-team, I directly knew why Erik called the HTS-team "overpowered". The number of times that you, **Flip**, solved the HPLC and GC problems are (un)fortunately not countable on two hands anymore. I also enjoyed all the Friday drinks, dinners and activities that you always joined. **Ilja**, thank you for helping me out with molecular biology stuff when the time pressure kicked in during the last months of my PhD. Every time I walked into your office to ask you another 'just one favour' was never declined. **Ewout**, you are a very skilled scientist in microbiology and biotechnology. You were always open for helping me out, whether it was related to running tall tubes, growth profiler experiments or taking that "one shake flasks measurement" (that never stayed at one sample…). I really enjoyed our trip to South-Africa for the ICY conference. It was a great experience to explore Cape Town, go on the whale boat trip and to spot the Big Five together with you. Thanks for all the support you gave me, especially in my final PhD year and I hope we stay friends. All of you are great assets of the HTS-team and I very much enjoyed working with all of you.

The collaboration with our industrial partner Heineken, especially **Viktor** and **Tom**, has been very fruitfull. Thank you for all the interesting and fun meetings, the opportunity to visit the Heineken pilot brewery, your critical view on manuscripts and the in-depth discussions we have had over the past years.

I would also like to extend my gratitude to the members of my thesis committee, **prof. dr. M. Ottens**, **dr. C.E. Paul**, **prof. dr. J.H. de Winde**, **dr. V. Boer** and **prof. dr. P. Branduardi** for your time and interest in my research. I am looking forward to the scientific debates and discussions during my defence.

While I learned from my supervisors, I also learned from students that I had the privilege to supervise for their Bachelors or Master thesis. **Diederik**, **Harshitha**, **Jimme**, **Jonah**, **Matic**, **Petrik**, **Roos** and **Siebe**: thank you for contributing to my PhD project. Your dedication, involvement and interest in the various research topics contributed not only to the science itself, but also to my personal development as supervisor. I really enjoyed to see the personal growth that each of you achieved during your time at IMB. Something equally enjoyable was your involvement in the IMB group outside working hours during the Friday drinks, the BBQ, the beer brewing festival and many other activities. Thank you for your trust in me and I will follow your career paths. Let's stay in touch.

During my bachelor and master thesis project that I performed at IMB, I was already taught the basic essentials, such as working in the IMB labs and guidelines on writing a thesis, and for that I thank my supervisors **Sofia** and **Paola**. **Sofia**, your enthusiasm towards science stimulated me to pursue a PhD. **Paola**, thank you for entrusting me

with finishing up the work you started on the "gEL DNA methodology" that resulted in a publication and formed a chapter of my dissertation. **Arthur**, although we were not employed at the same time, I would like to thank you for your wise words and advice from time to time, and of course for your well-written dissertation that I probably have read more often than anyone else by now.

The industrial microbiology group changes in composition due to the nature of PhD and Postdoc trajectories and careers. Therefore, the group picture of IMB at the start of my journey looks extremely different than the most recent one, but it has ever felt the same. **Sanne**, I was truly overwhelmed when you asked me the honour of being your paranymph. I very much appreciated the trust you gave me to prepare your cabaret together with Sophie, which was great fun. You are an open minded, critical researcher, a very patient, energetic teacher, a trustworthy, honest colleague, and, most importantly to me, you are a true friend! I am looking forward to the memories we will add in the future to the 'masker-avondje' with dinner, skiing holiday and parties we have been to. **Sophie**, I got to know you better during COVID19-times when we were the "last-remaining-online-4", together with Charlotte and Aafke, and when we were finally allowed into the lab, the both of us were privileged with the task to set up the laboratory upstairs for crash-courses molecular biology and other lab-related workshops for students. Since my first few months as PhD candidate, we hung out together more and more with regular 'gin&tonic chillings' at De Gist, Friday drinks at t' Klooster, 'Vroeg Pieken' parties at the 'Steck' and dinners with Job and Yael. I was very happy and honoured to be your paranymph, together with Aafke, and enjoyed preparing your cabaret. The sailing trips in Zeeland and the winter sport holiday in Austria were great fun and I am already looking forward to the adventures to come. Thank you for being part of my life and being a close friend!

It is a true privilege to have two friends by my side on probably one of the most important days of my professional career. **Céline**, thank you for being such an amazing friend, for your unwavering support and for always being there for me. The way you combine the two research groups with unlimited positive energy is something I admire. Thank you for all the fun moments we shared and I hope many more will follow in the future. **Marieke**, I am so thankful for the laughter and good times we have shared together. Your listening ear, understanding of emotions and encouragements, especially in the final months of my PhD, were essential to bring this thesis to the finish line. Your positive energy and open-minded attitude are very contagious. You've been there for me through thick and thin, and I will always cherish our friendship.

Anna, thank you for all your 'I-am-such-a-knowledgeable-fourth-year-PhD-student-talks' when I just started by PhD. You have prepared me well for the final PhD year. I also enjoyed teaching the Master course together with you. Most of all, I appreciate all the enthusiasm and kindness you bring to people. **Jasmijn**, you are a very fun person

to have in a group and I enjoyed the chats we have had in the lab. **Jonna**, our common hurdle in dealing with knee and ankle injuries during a PhD kind of brought us closer together. I enjoyed the chats in the lab with you and the fun at parties. **Charlotte**, although our PhD projects itself did not allow us to collaborate on research lines, we have spent a lot of time working together. The ASBEVI conference trip to Edinburgh was also great fun and one of the highlights of my PhD! One of your outstanding character traits is that you always stay positive, even when the situations were not always in your favour. You are a great scientist with an inspiring personality. I wish you all the luck in Wageningen with your future career. **Aafke**, we had the pleasure of organising the one-and-only 'IMB-online-lab-retreat' together; and fun it was! Thanks for the dinners, game nights and other parties we have been to. Enjoy your journey in Denmark and lets stay in touch! **Mark**, although the time were employed simultaneously was short, I have learned from your vision on science. The conference in Cork was a lot of fun and I hope to see you at many more events! **Nicoló**, thanks for spicing up the lunchbreaks with your jokes. **Maxime** and **Koen**, your knowledge on proteomics and systems biology has enriched the group is terms of science. I really enjoyed working with you and our chats in the lab. **Eline**, **Mario**, **Raúl**, **Thomas** and **Wijb**, thank you for your answering all my questions when I started my PhD journey.

Denzel, we have collaborated on many different project lines of which I hope that some may end up in your PhD thesis. I am looking forward to the day we can finally pop the champagne to celebrate the hard work. **Maartje**, when you joined IMB as PhD candidate, I was quite overloaded with different research lines. Hopefully, a few can reach the finish line thanks to your help. I wish you good luck with finishing your PhD. **Iris**, thanks for all your contributions to the meetings we have had together.

Sagarika, It was a pleasure sharing in the office together. Besides the hard work obviously, we also spent quite some time chitchatting. Teaching the Metabolic Reprogramming course together was fun and I am confident that you will get the bubbles to work! **Tobias**, your trains of thought have often brought different viewpoints to discussions or even completely new topics. Your energy is very positively contagious. **Rozanne**, since your first day at IMB you blend into the group like a natural and your positive attitude is very enjoyable to have around me. **Tom**, you are a true movie star! If you weren't such a bright scientist, I would have recommended a role at Hollywood. **Luca**, thanks for your note of sarcasm during the lunchbreaks and good luck with all your PhD plans. **Miriam** and **Rosalie**, you both have a very positive personality that is very enjoyable to have in the group. **Minke**, although we haven't worked much together, the karaoke-party was a night that I won't forget. **Effie**, your critical viewpoint on science is appreciated and you are a very kind person to have in the group. **Elynor**, **Marc**, **Shree** and **Yanfang**, it is great to have you as PostDocs in the group. Good luck with all your science and career plans! Thanks to **Michelle** and **Susan** for keeping IMB organized. The puzzle to arrange meetings according to all the diverse

and scattered schedules is a big challenge.

When I joined IMB as PhD candidate, the group was very open and welcoming. I hope this experience was and is the same for the current IMB group and that I contributed to this as well. Many thanks to **all of IMB** for the great atmosphere, the inspiring working environment, the input during the weekly work discussions, the enjoyable Friday drinks after lab cleaning, the memorable lab retreats, the unforgettable conference trips, and the fun activities outside work.

I am privileged with a very close group of friends outside the work environment. **Lisa**, het is een voorrecht om een vriendin te hebben als jij. Ik wil je bedanken voor de aandacht, de zorg en het geduld dat je met me hebt gehad, in tijden van vreugde en moeilijkere momenten. **Lisa**, **Manon**, **Hugo**, **Delia**, **Isabelle**, en alle andere **WVZ-zwemmaatjes**, iedere week heb ik de mogelijkheid om met jullie tijdens de zwemtraining volledig te ontkoppelen van mijn onderzoek en alle stress en frustraties eruit te zwemmen. **Maik**, dankjewel voor het uitlenen van je fotografie skills en de tijd die je in de omslag van mijn proefschrift hebt gestopt. Ik ben erg blij met het resultaat! **WAVE Dames 1**, dankjewel dat ik iedere zaterdag mag aantreden als waterpoloster. Jullie zijn een geweldig team om onderdeel van te zijn! **Roos**, **Jolien**, **Jimmy** en **Noud**, ondanks dat we bij elkaar de deur niet platlopen, is de gezelligheid die jullie brengen bij onze dinertjes, spelletjesavond, mimosabrunches en theedrinksessies altijd waardevol voor mij. Roos, ik ben ontzettend trost op je doorzettingsvermogen. Jimmy, je bent gewoon een kanjer! Jolien en Noud, heel veel succes bij het afronden van jullie PhD. **Maud**, **Olmo**, **Bram** en **Roos**, goede vrienden zijn van onschatbare waarde en ik ben ontzettend dankbaar met jullie om mij heen. Jullie steun in de leuke en minder leuke tijden waardeer ik enorm, ook al zullen jullie dit misschien vanzelfsprekend vinden.

Lieve **Paul** en **Nathalie**, **Omri**, **Sira** en **Noam**, ik ben ontzettend blij met jullie als schoonfamilie. Dankjewel dat ik altijd bij jullie terecht kan.

Lieve **pap** en **mam**, vanaf de dag dat ik geboren ben heb ik alleen maar onvoorwaardelijke liefde van jullie gekregen. Jullie support hebben gemaakt wie ik op de dag van vandaag ben. Jullie steunen mij in de keuzes die ik maak, en helpen mij bij het bereiken van mijn doelen. Zonder jullie had ik het niet kunnen en willen doen. Lieve **oma**, je staat altijd voor me klaar en bent mijn grote steun als het gaat om mijn studie en promotie. Ik hoop dat ik je een trotse oma heb gemaakt om de persoon die ik ben. Lieve **Rolan**, op jou kan ik altijd rekenen. Onze band is zo ontzettend hecht en sterk en dat wil ik voor geen goud anders zien. Ik ben trots op hoe jij alles in het leven aanpakt. Je maakt me een trotse zus!

Lieve **Yael**, ik wil je bedanken voor elke lach, elke traan, elke knuffel en genegenheid op mooie en minder leuke momenten. Je bent mijn rots in de branding. Je zorgt ervoor dat mijn wereld altijd sprankelt. Ik ben trots op je. Ik hou van je!

Curriculum vitae

Nicole Xanthe Bennis was born on April 13th 1996 in Zoetermeer, The Netherlands. In 2008, Nicole started pre-university (VWO) education at Het Oranje Nassau College in Zoetermeer and at Het Picasso Lyceum in Zoetermeer. She followed the pre-university program of Molecular Science and Technology at Leiden University. Nicole graduated in 2013 with 'Socrates honours'. After being inspired by science courses, she started the BSc joint degree program Life Science and Technology at Leiden University and Delft University of Technology. Nicole finalized the three-year education program with a bachelor end project in the Industrial Microbiology section at the Delft University of Technology under supervision of Prof. dr. ir. Pascale Daran-Lapujade and dr. Sofia Dashko. The project focused on the selective removal of synthetic chromosomes from engineered *S. cerevisiae* strains using CRISPR-Cas9. After obtaining the BSc degree with honours in 2017, Nicole continued her education with the two-year MSc program Life Science and Technology at Delft University of Technology with Cell Factory as specialization track and electives in fermentation technology, environmental biotechnology, protein engineering and green chemistry. In 2018, Nicole joined the TU Delft iGEM team, with whom she developed a targeted sequencing method for the detection of gene doping in samples of sport athletes and was awarded two awards, the gold medal and nominations for six prize categories at the international Jamboree in Boston, MA, USA. She performed the nine-month master thesis project in the Industrial Microbiology section at the Delft University of Technology under supervision of Prof. dr. ir. Pascale Daran-Lapujade and dr. Paola Randazzo, were she developed a CRISPR-Cas9-based tool to generate genetic diversity in *S. cerevisiae* strains. She completed her Master's program with an industrial internship at DAB located at PlanetB.io in Delft under supervision of dr. Kirsten Herben-Steinbusch and Fabienne Feskens-Snoeck. The company DAB provides a technology platform to enable cost-effective biomanufacturing of inhibitory or unstable products. In April 2020, Nicole embarked on a PhD trajectory in the Industrial Microbiology section at Delft University of Technology under supervision of Prof. dr.ir. Jean-Marc G. Daran and Prof.dr. Jack T. Pronk, where she studied the genetics of hybrid yeasts, developed new genetic engineering tools for yeasts and explored metabolic engineering of lager brewing yeasts for brewing applications. The results of this project are presented in this dissertation.

List of publications

Randazzo P, **Bennis NX**, Daran JM, Daran-Lapujade P (2021). gEL DNA: A cloning- and polymerase chain reaction-free method for CRISPR-based multiplexed genome editing. *CRISPR Journal*. 4(6):896-913. doi: 10.1089/crispr.2020.0028.

Bennis NX, Kostanjšek M, van den Broek M, Daran JG (2022). Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts. *New Biotechnology*. 25(76):49-62. doi: 10.1016/j.nbt.2023.04.001.

Bennis NX, Anderson JP, Kok SMC, Daran JG (2023). Expanding the genome editing toolbox of *Saccharomyces cerevisiae* with the endonuclease *Er*Cas12a. *FEMS Yeast Research*. 4(23):1-14. doi: 10.1093/femsyr/foad043.

van den Broek M, Ortiz-Merino RA, **Bennis NX**, Wronska AK, Hassing E-J, Daran-Lapujade P, Daran JMG (2023). Draft genome sequence of the *Saccharomyces cerevisiae Spy*Cas9 expressing strain IMX2600, a laboratory and platform strain from the CEN.PK lineage for cell-factory research. *Microbiology Resource Announcement*. 22. doi: 10.1128/mra.00550-23.

Bennis NX, Bieseman J, Daran JG. (2024). Unlocking lager's flavour palette by metabolic engineering of *Saccharomyces pastorianus* for enhanced ethyl ester production. *Metabolic Engineering*. 85:180-195. doi: 10.1016/j.ymben.2024.08.002.

Bennis NX, HI Hanji, van den Broek M, Daran JMG (2024). Chromosome level genome assembly and annotation of Hanseniaspora mollemarum CBS 18055 strain. *Submitted for publication in Microbiology Resource Announcement*.

Selected conference contributions

Bennis NX. gEL DNA: A cloning- and polymerase chain reaction-free method for CRISPR-based multiplexed genome editing. [poster presentation] ASBE V. November 2-4 2020. Delft, The Netherlands (online).

Bennis NX. gEL DNA: A cloning- and polymerase chain reaction-free method for CRISPR-based multiplexed genome editing. [oral presentation] ICY15 meets 30 ICYGMB. August 23-27 2021. Vienna, Austria (online).

Bennis NX. Expanding the genome editing toolbox of *Saccharomyces cerevisiae* with the endonuclease *Er*Cas12a. [poster presentation] ASBE VI. November 2-4 2022. Edingburgh, United Kingdom.

Bennis NX. Expanding the genome editing toolbox of *Saccharomyces cerevisiae* with the endonuclease *Er*Cas12a. [oral presentation] Mycology Meeting. November 25 2022. Utrecht, The Netherlands.

Bennis NX. Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts. [poster presentation, poster prize] PYFF8. June 5-8 2023. Cork, Ireland.

Bennis NX. Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts. [oral presentation] ECB. June 30 - July 3 2024. Rotterdam, The Netherlands.

Bennis NX. Unlocking lager's flavour palette by metabolic engineering of *Saccharomyces pastorianus* for enhanced ethyl ester production. [oral presentation] ICY16. September 29 - October 3 2024. Cape Town, South Africa.