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DOI
10.4233/uuid:0c847298-0007-4922-aff4-00beb248d664

Publication date
2019

Document Version
Final published version

Citation (APA)
Brickwedde, A. (2019). *Maltose and maltotriose metabolism in brewing-related Saccharomyces yeasts*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:0c847298-0007-4922-aff4-00beb248d664

Maltose and maltotriose metabolism in brewing-related *Saccharomyces* yeasts

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
Wednesday 6 March 2019 at 15:00 o'clock

by

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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. The project was financed by the Seventh Framework Programme of the European Union in the frame of the SP3 people support for training and career development of researchers (Marie Curie), Networks for Initial Training (PITN-GA-2013 ITN-2013-606795) YeastCell.

Contents

Summary

Grain-based fermented beverages have been produced since, several millennia BCE, human societies developed a settled lifestyle based on agriculture. In the 15th century CE, lager type beers similar to the present-day ones first emerged in Central Europe. Lager beer is produced from barley, hops, and water. Conversion of carbohydrates from grains to ethanol and CO_2 is catalysed by S*accharomyces pastorianus* yeasts. Those yeasts are hybrids of *S. cerevisiae* and *S. eubayanus* that, through their ability to efficiently ferment the predominant wort sugars at low temperatures, combine traits from both parents. Remarkably, *S. pastorianus* strains have exclusively been isolated from man-made environments related to lager beer brewing, and not from natural ecosystems, suggesting that they have arisen and evolved in breweries.

Maltose and maltotriose are taken up by *S. pastorianus* via a set of proton symporters, the structural genes for which are derived from each of its parents and subsequently evolved throughout the process of brewing yeast domestication. In contrast to the well-studied maltose uptake and hydrolysis in *S. cerevisiae*, knowledge on transport and metabolism of this α-glucoside by *S. eubayanus* is limited. The study described in **Chapter 2** of this thesis systematically investigates the functionality of all annotated genes encoding potential maltose transporters present in the *S. eubayanus* type strain CBS12357, namely *MALT1*, *MALT2*, *MALT3*, and *MALT4*. First, using Oxford Nanopore Technology's MinION long-read sequencing platform, a near-complete genome sequence of this strain was assembled, which provided, amongst other new genomic features, complete sequence information of the four *MAL* loci. All *S. eubayanus MALT* genes were then separately expressed in a maltose-transport-negative *S. cerevisiae* strain background. The four genes were shown to support growth on synthetic medium with maltose as sole carbon source, thereby confirming their ability to encode functional maltose transport proteins. In addition to the heterologous expression of single transporters in *S. cerevisiae*, *MALT* genes were systematically deleted in *S. eubayanus* CBS12357 using CRISPR-Cas9 based genome editing to explore their contribution to maltose utilization in their natural genetic context. Deletion of *MALT2* and *MALT4*, which share 99.7% sequence identity, led to a strain which did not grow on maltose. Conversely, strains carrying deletions in both *MALT1* and *MALT3* showed a similar physiology as the initial strain. The conclusion that Malt2 and Malt4 are the only maltose transporters active in CBS12357 was further supported by increased expression levels of *MALT2* and *MALT4* in cultures grown on maltose. In contrast, *MALT1* and *MALT3* showed negligible expression levels in maltose-grown cultures.

In **Chapter 3** of this thesis, two major characteristics of lager yeasts that are relevant for the success of the brewing process, cryotolerance and the ability to ferment maltose and maltotriose, are investigated in an interspecies hybrid that was constructed via mass mating of a haploid derivative of the *S. eubayanus* type strain CBS12357 and a

haploid *S. cerevisiae* laboratory strain of the CEN.PK family. The performance of the constructed hybrid, strain IMS0408, was then compared to those of its parents in anaerobic batch cultures grown on different media and at different temperatures. While *S. eubayanus* displayed significantly higher growth rates than *S. cerevisiae* in anaerobic batch cultures below 25 °C, the laboratory hybrid IMS0408 performed as well as the best parent or even better at most tested temperatures. In contrast to its *S. eubayanus* parent, the hybrid strain was further able to consume maltotriose, the second most abundant sugar in wort, in cultures grown on sugar mixtures. This observation showed how acquisition of the *S. cerevisiae* genome contributed an important brewing related characteristic of the hybrid. The hybrid strain IMS0408 showed a best parent heterosis in two major characteristics that are relevant in the brewing environment. This heterosis illustrates how an early, spontaneous *S. pastorianus* lager brewing hybrid might have outcompeted other *Saccharomyces* species, including its parental ones, under the low-temperature, high-maltotriose conditions of lager fermentation processes.

While maltose is fermented rapidly by most *S. pastorianus* strains, maltotriose is often taken up much slower than maltose and in many cases not completely fermented. Such suboptimal maltotriose fermentation kinetics compromise the stability and economics of brewing processes. **Chapter 4** explores whether evolutionary engineering can be applied to improve the kinetics of maltotriose fermentation and, in particular, of the transport of this oligosaccharide across the yeast plasma membrane. Evolutionary engineering was chosen over targeted genetic modification because of the genetic complexity of maltose and maltotriose metabolism, which made it difficult to a priori identify targets for genetic modification. Moreover, in view of limited customer acceptance for beverages made with genetically modified organisms (GMOs), it is attractive for industrial applications that strains obtained by evolutionary engineering approaches are not considered to be GMOs. The lager brewing strain *S. pastorianus* CBS1483 was grown in carbon-limited chemostat cultures on a maltotriose-enriched mixed-sugar medium. After 80 generations of selective growth, a reduction by 70 % of the residual maltotriose concentration was observed in four independent bioreactor evolution experiments. Cell lines isolated from evolved cultures were characterized in 1L laboratory, 2L tall-tube, as well as in 1,000L pilot scale fermentations. Of these cell lines, selected single-colony isolate IMS0493 resembled the evolved culture in steady-state cultures by yielding a lower residual maltotriose concentration and higher biomass concentration than the parental, non-evolved strain CBS1483. Moreover, batch cultures of the evolved strain on wort as well as synthetic medium showed a much more complete conversion of maltotriose than observed with the parental strain. In uptake studies with radioactive, ^{14}C-labelled sugars, the evolved strain showed a 4.8-fold higher V_{max} for maltotriose compared to the non-evolved strain (23.5 and 4.9 $\mu mol \cdot min^{-1} \cdot (g_{dry\ biomass})^{-1}$, respectively) while the transport capacity for maltose was similar in both strains. In industrial pilot-scale fermentations, the evolved strain IMS0493 also clearly expressed the characteristics acquired during adaptive laboratory evolution. Maltotriose

concentrations in the broth at the end of the fermentation were significantly lower than found with the reference strain and ethanol concentrations were 6 % higher than in fermentations with the non-evolved strain CBS1483. Aroma profiles of bottled beer produced with the evolved isolate met the quality standards for lager beers. Successful transfer of the improved fermentation kinetics of the evolved *S. pastorianus* strain to an industrial scale demonstrated the relevance of evolutionary engineering strategies for industrial applications, and in particular for lager beer brewing.

Samenvatting

Op granen gebaseerde dranken worden al geproduceerd sinds de mensheid, duizenden jaren geleden, een op landbouw gebaseerde levensstijl heeft ontwikkeld. In de 15de eeuw zijn lagerbieren, vergelijkbaar met het huidige pils, ontstaan in Centraal-Europa. Lagerbier wordt geproduceerd uit gerst, hop en water. De omzetting van koolhydraten uit de granen naar ethanol en CO_2 wordt gekatalyseerd door *Saccharomyces pastorianus*-gisten. Deze gisten zijn hybriden van *S. cerevisiae* en *S. eubayanus* en hebben door de hybridisatie eigenschappen van beide ouders verkregen. Dit leidde onder andere tot het vermogen om efficiënt wortsuikers om te zetten bij lage temperatuur. Opmerkelijk is dat *S. pastorianus* stammen uitsluitend uit door mens gemaakte omgevingen geïsoleerd zijn en niet uit natuurlijke ecosystemen. Deze waarneming duidt op een evolutionaire oorsprong van deze gisten in brouwerijen.

De opname van maltose en maltotriose, de twee meest voorkomende suikers in wort, door de gistcel gebeurt via een set van proton-symporters in het plasmamembraan. De genen die in *S. pastorianus* voor deze transporters coderen zijn afkomstig van beide voorouders (*S. cerevisiae* en *S. eubayanus*) en hebben, gedurende domesticatie, gespecialiseerde functies ontwikkeld. In tegenstelling tot het goed bestudeerde maltosetransport in *S. cerevisiae*, is de kennis over α-glucosidetransport en -stofwisseling in *S. eubayanus* beperkt. **Hoofdstuk 2** beschrijft een studie naar de functionaliteit van alle geannoteerde genen van de *S. eubayanus* typestam CBS12357 die potentieel voor maltosetransporters coderen (*MALT1*, *MALT2*, *MALT3* en *MALT4*). Om te beginnen werd in dit Hoofdstuk, gebruikmakend van het Oxford Nanopore Technology MinION longread sequencing platform, een vrijwel volledige genoomsequentie van deze typestam geassembleerd en geannoteerd. Deze annotatie leverde, naast andere nieuwe inzichten in genetische kenmerken van deze gist, een complete sequentie van alle vier de *MAL*-loci op. De *MALT* genen werden vervolgens apart in een maltose-transport-negatieve *S. cerevisiae*-stam tot expressie gebracht. Omdat alle stammen op synthetisch medium met maltose als enige koolstofbron groeiden, kon geconcludeerd worden dat alle vier de *MALT* genen voor functionele maltosetransporteiwitten coderen. Naast deze heterologe expressie van individuele transporters in een *S. cerevisiae*-stam, werden de *MALT*-genen in *S. eubayanus* CBS12357 met behulp van CRISPR-Cas9 systematisch verwijderd om hun bijdrage aan de maltose-omzetting in hun natuurlijke genetische context te onderzoeken. Deletie van *MALT2* en *MALT4*, die een 99,7 % identieke sequentie hebben, leidde tot een stam die niet op maltose groeide. Daarentegen lieten stammen met deleties in *MALT1* of *MALT3* een vergelijkbaar fenotype zien als de controlestam. De conclusie dat Malt2 en Malt4 de enige actieve maltosetransporters in CBS12357 zijn, werd ondersteund door de verhoogde expressie-niveaus van *MALT2* en *MALT4* tijdens groei op maltose. *MALT1* en *MALT3* vertoonden daarentegen minimale expressieniveaus in op maltose gekweekte cultures.

In **Hoofdstuk 3** van dit proefschrift worden twee eigenschappen van een hybride gist, die relevant zijn voor het succes van het brouwproces (cryotolerantie en het vermogen om maltose en maltotriose te assimileren, onderzocht. De hybride werd verkregen door "mass mating" tussen haploïde spores, afkomstig van de *S. eubayanus* type stam CBS12357, en een haploïde *S. cerevisiae* stam uit de CEN.PK familie. De prestatie van deze nieuw construeerde hybride, stam IMS0408, werd vergeleken met die van beide ouderstammen in anaërobe batchcultures op verschillende kweekmedia en bij verschillende temperaturen. Terwijl *S. eubayanus* een significant hogere groeisnelheid had dan *S. cerevisiae* in anaërobe batchcultures bij temperaturen beneden de 25 °C, groeide de hybride minstens even goed als en bij sommige temperaturen zelfs beter dan de beste ouder. In tegenstelling tot de *S. eubayanus*-ouder was de hybridestam in staat om maltotriose, de op maltose na meest voorkomende suiker in wort, op te nemen uit media die een mengsel van suikers bevatten. Dit verduidelijkt hoe de verwerving van het *S. cerevisiae* genoom door de hybride stam bijdraagt aan deze belangrijke, voor het bier brouwen relevante, eigenschap. In de hybridestam IMS0408 treedt dus voor twee uiterst belangrijke eigenschappen van lagerbiergisten, een heterosis-effect op. Deze heterosis illustreert hoe een *S. pastorianus* hybride, in de koude suikerrijke omgeving van lagerbierfermentatie, andere *Saccharomyces* soorten, waaronder ook de ouders van de hybride, heeft kunnen verdringen.

Terwijl maltose snel door de meeste *S. pastorianus*-stammen omgezet kan worden, wordt maltotriose vaak veel langzamer en/of onvolledig opgenomen en vergist. Een dergelijke suboptimale kinetiek van maltotriose vergisting beperkt de stabiliteit en winstgevendheid van het brouwproces. **Hoofdstuk 4** bestudeert de mogelijkheid om gerichte evolutie in het laboratorium te gebruiken voor het verbeteren van de kinetiek van maltotriose vergisting, in het bijzonder het transport van deze oligosacharide over het celmembraan. Er werd gekozen voor gerichte evolutie in plaats van genetische modificatie vanwege de genetische complexiteit van maltose- en maltotriose-stofwisseling. Deze complexiteit maakt het moeilijk om vooraf targets voor genetische modificatie precies te definiëren. Bovendien is het, in verband met de beperkte acceptatie van genetisch gemodificeerde organismen (GGO's) door consumenten, aantrekkelijk voor industriële toepassingen dat stammen die met behulp van gerichte evolutie ontwikkeld zijn, niet als GGO's worden beschouwd. In deze studie werd de lagerbiergist stam *S. pastorianus* CBS1483 gekweekt in koolstof-gelimiteerde continu cultures, op een kweekmedium dat naarst een mengsel van suikers vooral veel maltotriose bevatte. In vier onafhankelijke evolutie-experimenten in bioreactoren was de residuele maltotrioseconcentratie na 80 generaties van selectieve groei met 70 % verminderd. Uit deze evolutiekweken geïsoleerde cellijnen werden in 1-liter bioreactoren, in 2-liter tall-tubereactoren en in 1.000-liter "pilot-scale" fermentaties gekarakteriseerd. Een uit de evolutie-experimenten geïsoleerde giststam, IMS0493, bleek in chemostaat-cultures een lagere residuele maltotriose concentratie en een hogere biomassa-opbrengst te vertonen dan de ouderstam CBS1483. Daarnaast toonde deze geëvolueerde stam, in vergelijking

met de ouderstam, een meer complete omzetting van maltotriose in batchcultures op zowel synthetisch medium als ook op wort. In suikeropname studies met radioactieve, ^{14}C-gelabelde suikers had de geëvolueerde stam een 4,8 keer hogere V_{max} voor maltotriosetransport dan de controlestam (respectievelijk 23,5 en 4,9 $\mu mol \cdot min^{-1} \cdot (g_{droge\ biomassa})^{-1}$) terwijl de transportcapaciteit voor maltose in beide stammen gelijk was. Ook in de industriële pilot-schaal vergisting liet de geëvolueerde stam IMS0493 eigenschappen zien die tijdens de laboratoriumevolutie verkregen waren. Maltotriose concentraties aan het eind van de fermentatie waren significant lager en ethanol concentraties 6 % hoger dan aan het eind van de controlefermentatie met stam CBS1483. Aromaprofielen van gebotteld bier dat met de verbeterde stam geproduceerd werd voldeden aan de kwaliteitseisen voor lagerbier. De relevantie van gerichte evolutie als strategie werd aangetoond door de succesvolle verbetering van de transportkinetiek in een geëvolueerde *S. pastorianus*-stam op industriële schaal. Dit resultaat is in het bijzonder belangrijk en toepasbaar voor lagerbierbrouwprocessen.

Chapter 1: Introduction

Fermented beverages and the role of yeast in brewing – a historical context

Microbial fermentation has been used for the production of alcoholic beverages since the dawn of human civilization. Along with the domestication of plants and animals, the history of brewing can be traced back to at least 10,200 BCE [1]. The first fermented beverages were made from fruit- and tree juices, which spontaneously undergo alcoholic fermentation upon storage [2]. Our fruit-eating (frugivorous) primate ancestors, feeding on ripe, partially fermented fruits, demonstrate a plausible evolutionary origin of the consumption of fermented plant sources and of human alcoholism [3]. This observation suggests that human consumption of fermented natural resources, in which fermentation did not only produce ethanol and/or lactic acid but also improved characteristics such as flavour, nutritional value and stability, has likely occurred even earlier than is now documented by archaeological research.

Microorganisms present on the surface of ripe fruit (including yeasts, molds, and bacteria) convert a small fraction of the fruit sugars into ethanol. Consequently, it is likely that all frugivores are regularly exposed to low doses of this compound. Ethanol and other volatiles released during microbial fermentation potentially served as localization signals for food sources and as appetitive stimulants [4], thus causing an evolutionary advantage for fruit-eating animals with a sensory bias towards ethanol in their feeding strategies. Studies have shown that, along with consumption of fermented plants, adjustments in the metabolism of frugivorous organism arose. For example, environmental exposure to ethanol has been shown to result in adaptation of the expression of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in *Drosophila melanogaster* [5] as well as in humans [6].

The shift from a hunter-gatherer lifestyle to human societies based on agriculture was to a large extent driven and enabled by cultivation of grasses and grains [7]. Widespread implementation of processes for microbial fermentation of these resources led to development of bread and beer as staple sources of calories and nutrients [2]. The first evidence for grain-based fermented beverages was found in China [8] where, already around 7,000 BCE, alcoholic beverages were prepared from rice, fruit and honey. Usage of grains for producing nutritional, fermented beverages has likely developed independently on several continents. Depictions of the consumption of fermented beverages (such as shown in Figure 1) and of the beer brewing process found on walls of

Egyptian tombs indicate that beer was produced in the Middle East around 3,500 BCE [9]. In Europe, cultivation of crops began about 6,000 years ago by Nordic, Celtic and Germanic tribes [1]. While southern European peoples (including Greeks and Romans) traditionally were determined wine drinkers, British, Spanish, and German peoples preferred ale-type beers. The usage of hops in beer brewing in Europe was first documented in medieval times, when hops from China were supplemented to beers produced in monasteries for trade between the European continent and the British isles to ensure stability during transport [1, 2]. Hops serve as bittering agents, add a spicy flavour to the otherwise sweet beer and also exhibit antimicrobial properties and thus serve as natural preservatives [10].

In medieval times, brewing was increasingly professionalized in cities in Northern Europe. Guilds such as the Hanseatic league (founded in 1241) [11] were formed, and a few centuries later marine empires like the Dutch and British shipped beer overseas to their colonies [1]. With the enforcement of the Bavarian Purity Law ("Reinheitsgebot") in 1516 [12], the first regulation of this kind applying to a whole state, beer production was standardized and product quality improved by restricting the allowed raw materials to barley malt, water, and hops [1]. To produce beer, *Saccharomyces cerevisiae* ale-brewing yeasts were traditionally used. These ale yeasts rise to the top of the vessel at the end of the fermentation and are therefore referred to as top fermenting yeasts. A new type of beer, produced through bottom fermentation (in which the yeast sinks to the bottom of the fermentation vessel once sugars have been completely fermented), arose in the 15th century after brewing in the summer months was prohibited in Bavaria. Lager beer (from the German "lagern" = to store) produced in winter was stored in cold cellars throughout the year to minimize spoilage. Bottom fermentation was first documented for production of dark, hopped beer in Bavaria. Later, pale, hopped Pilsner, made by bottom fermenting yeast and created in 1842 in Bohemia, in the present-day Czech Republic, quickly gained popularity in Europe. This style of beer production is still used for the present-day produced lager beer (for further details on the process of beer brewing and ale and lager

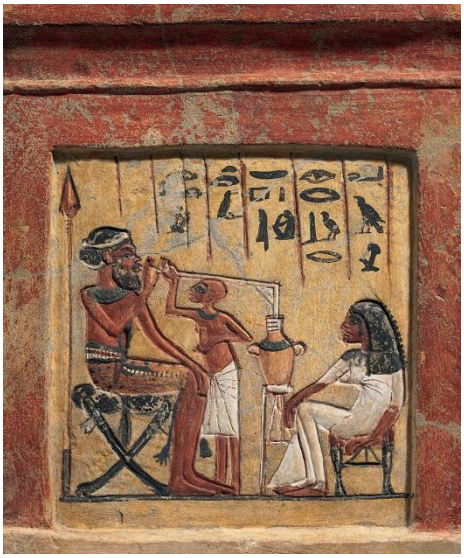

Figure 1: Funerary stela from El Amarna, Egypt (1351 BCE - 1334 BCE) with a representation of a Syrian mercenary drinking wine through a drinking tube. (Staatliche Museen zu Berlin - Ägyptisches Museum und Papyrussammlung, Inv. Nr.: 14,122, Foto: Sandra Steiß, issue date 12.07.2018)

beer styles, see below). Until the 19th century, however, mankind remained unaware of the key role played by microorganisms in beer brewing.

In the 18th and 19th century, the generation and implementation of scientific knowledge facilitated development of new brewing equipment and technologies. After improvements in microscopy, between 1836 and 1838, three independent scientists showed that the yeast used in the production of beer and wine in fact represented living organisms [13]. Louis Pasteur analysed and established the relevance of yeast for alcoholic fermentation, the physiological phenomenon of fermentation in yeast, and differences between aerobic and anaerobic growth of these microorganisms on sugar-rich medium in the years 1855-1875 [14]. Pasteur already recommended the use of pure yeast cultures for brewing purposes (1876) to ensure a stable quality of the final product but did not himself succeed in obtaining a yeast culture free of other microbial contaminants. Pure yeast cultures were first isolated in 1883 by Christian Hansen at the Carlsberg brewery and in 1886 by Hartog Elion at the Heineken brewery [15]. From the mid-19th century on, brewing was widely recognized as an economically important discipline in science and technology and, as such, taught at universities. In 1865, a brewing curriculum was first offered at the Technical University Weihenstephan in Munich, followed by the University of Berlin in 1888.

The 20th century saw an explosive growth in the industrialization of brewing and in the consumption of beer around the world. During this 'globalization' of beer consumption, lager-style beers gradually became the predominant type of beer. Whereas, for most of human history, beer types and preferences had shown large regional diversity, a limited number of large brands of lager beer now accounts for over 80 % of the global beer market. In 2016, 1.96 billion hectoliters of beer were produced worldwide (https://www.statista.com) of which lager beer accounted for 89%. In recent years, consumers show an increasing interest in beer diversity, which has contributed to a rapid increase in the numbers of 'craft breweries' that produce comparatively small volumes of specialty beers. The craft beer sales in the US grew 5% in 2017, reaching 12.7% of the total market volume, with the craft beer market accounting for 23.3% dollar sales of the US beer market [16].

The brewing process – from grains to beer

The processing of grains and the production of wort

The beer production process is based on extraction and hydrolysis of carbohydrates from cereal grains and their subsequent fermentation by yeast to CO_2 and ethanol (for an overview see Figure 2). The grains used for beer brewing (mostly barley or wheat) are rich in carbohydrates, predominantly in the form of starch [17]. To facilitate access of brewing yeast to these carbohydrates, the starch is hydrolysed to fermentable

sugars in a process called malting. In a first step, called steeping, water is added to the grains, which stimulates their germination. The sprouting plant cells produce enzymes that enable hydrolysis of β-glucans and arabinoxylans localized in the cell wall, some protein and starch from the plant endosperm (β-glucanases, xylanases, proteinases and carboxypeptidases and α-amylases, respectively) [18]. After four to five days, reduction of the moisture content at a high temperature, in a process step called kilning, stops the growth of the seedlings, provides "malty" flavour and colours and stabilizes the malt. During the malting process, lipids released from the plant endosperm are oxidized and broken down into aldehydes. These aldehydes are responsible for a fresh, grassy taste but can also give the beer a solvent-like off flavour when present in too high concentrations [19]. After malting (consisting of the three steps described above), the germinated, dried grains are milled in order to extract carbohydrates from the crushed grains. The milled grains are then mixed with water and heated (mashing), which promotes amylase activity and, consequently, the hydrolysis of starch into simpler sugars such as maltose and maltotriose [20]. The sweet broth is then separated from the spent malt (lautering). The resulting liquid, called wort, is composed of sugars that can be fermented by yeast, dextrins, proteins and other compounds of importance for beer flavour originating from the grains [21]. Growth of yeast in the wort, which largely occurs under anaerobic conditions, is accompanied by the formation of ethanol, CO_2 and a multitude of aroma compounds [22]. Wort amino acids are a main nitrogen source for yeast and important for the production of higher alcohols via the Ehrlich pathway [337]. Ferulic acid and other hydroxyl cinnamic acids, originated from the plant cell wall, can be decarboxylated by many so called Phenolic Off Flavour Positive (POF^+) yeasts resulting in the production of phenolic off flavours [19].

While the composition of wort strongly depends on the malting and mashing process and on the type and harvest of grains used, the relative concentrations of the most important sugars in wort are rather constant. Approximately 10 % of the fermentable wort sugars is made up by polymeric α-glucosides (dextrins), glucose, fructose and sucrose. The α-glucosides maltose (50 – 60 % of the fermentable wort sugars) and maltotriose (10 - 20 %) represent the largest fraction of fermentable sugars [23, 24]. A (near) complete conversion (attenuation) of the wort sugars by yeast fermentation is a crucial requirement for reproducible, stable product formation.

Wort is boiled in order to kill bacteria and wild yeast. During boiling, hops, which are the flowers of the plant *Humulus lupulus* that are generally dried before use, are added to give bitterness and flavour to the beer (due to an isomerization of hop acids to bitter iso-α-acids [25]) and to balance the sweetness of the malt. During wort boiling, additional aldehydes can be formed from lipid oxidations and Maillard reactions [19]. Hopped wort is then cooled and prepared for fermentation. Prior to inoculation (pitching) of yeast, the wort is oxygenated. This brief oxygenation phase, which precedes the subsequent anaerobic fermentation phase, is crucial for the brewing yeast since it enables the oxygen-dependent synthesis of unsaturated fatty acids (UFAs) and sterols [26, 27].

Simultaneously, zinc, a co-factor for numerous yeast enzymes whose concentration in many worts is insufficient to support efficient fermentation, may be added [28-30]. As the solubility of oxygen in water is relatively low, wort is commonly oxygenated (not aerated) to achieve concentrations close to the maximum of 40 mg L^{-1} (in contrast to 6 mg L^{-1} for aeration) [26]. Management of wort oxygen not only has an impact on the rate and extent of fermentation but also on flavour and aroma profile of the final product.

Figure 2: Schematic representation of the beer brewing process. Carbohydrates from cereal grains are extracted through germination (malting), mixed with water and broken down into fermentable sugars (mashing). After separation from spent grains, hops are added to the resulting sweet broth (wort). Wort sugars are converted by brewing yeast into CO_2 and ethanol and after conditioning, the final product, beer, is ready.

Alcoholic fermentation and the brewery environment representing a harsh surrounding for the yeast

Pitching usually happens at an initial concentration of 1 million cells mL^{-1}, propagated in oxygenated wort, for every 1 °Plato (the Plato gravity scale is used to quantify the percentage of soluble material of the wort, mainly sugars [31]) of the wort [32].

Nowadays, the majority of beers are fermented with bottom-fermenting, cold-tolerant, *Saccharomyces pastorianus* lager brewing yeasts, which have the tendency to flocculate and sink to the bottom of the fermentation vessel at the end of fermentation. Optimal temperatures for lager beer fermentations were shown to be not higher than 15 °C, whereas ale fermentations are usually performed at temperatures between 20 and 30 °C [33] in accordance with the different temperature tolerances and optima of *S. cerevisiae* ale yeasts and *S. pastorianus* lager yeasts [34, 35]. To reduce the risk of contamination and selection of genetic variants that could negatively affect product quality, the yeast is commonly used for a maximum number of 5 – 10 brewing fermentation cycles through re-pitching of yeast produced in a previous cycle. In between fermentations, yeast is stored anaerobically at low temperature (usually 1 - 5 °C) [26, 36] and high ethanol concentrations from the preceding fermentation [37]. Prior to re-pitching, many brewers integrate an acid washing step, for example with a solution of phosphoric acid, to

minimize bacterial contamination [38]. During pitching, the yeast experiences a sudden shift from a cold, anaerobic environment in a storage tank to nutrient-rich, aerated wort at higher temperatures. Especially in very high gravity fermentations, osmotic pressure is an additional important stress factor [37], which the yeast cells have to counter by accumulating the compatible solutes glycerol and trehalose [39]. In addition to being exposed to a high osmolality, cells encounter an alternating hydrostatic pressure as mixing caused by CO_2 gas bubbles moves them through the large-volume brewing vessels. Already early in the fermentation process, the oxygen that was introduced at the start of the process rapidly gets depleted and the high concentrations of sugar (15 – 20 °Plato) are converted into, amongst other products, ethanol. As the ethanol concentration increases, and especially in very high gravity fermentations, it becomes an additional important stress factor which can have a negative effect on yeast performance [40]. Ethanol toxicity affects cell growth, viability, and overall fermentation performance [41]. The main targets of ethanol toxicity is the yeast cell membrane which, at high ethanol concentrations, undergoes a decrease in integrity [42]. High ergosterol and unsaturated fatty acid levels are key factors for ethanol tolerance [43] and the incorporation of oleic acid into the membrane counteracts the increase in fluidity caused by high extracellular ethanol levels [44, 45]. To counter an increased flux of protons inside the cell upon exposure to high ethanol levels, which would otherwise disrupt the proton-motive force, the activity of ATPases in the plasma membrane increases [46].

Flocculation

Especially at the end of the fermentation, a process called flocculation helps to protect cells against the inhibitory effects of high ethanol and also CO_2 concentrations [47]. Flocculation represents the association of individual cells into large, multi-cell aggregates, that rapidly sediment (hence the term 'bottom fermentation'). In brewer's yeasts, flocculation is caused by an interaction between flocculin proteins and cell surface mannans and is mediated by calcium ions [48]. The flocculins are blocked by bound wort sugars until the last phase of the fermentation. Yeast flocculation in beer brewing is a nonsexual, reversible (through the addition of EDTA and/or sugars) aggregation of cells into flocs and a relevant phenotype for the success of a brewing fermentation [47, 48]. The process of flocculation is controlled by so called *FLO* genes, mainly encoding flocculins, of which at least nine are known in brewing yeasts [49]. *FLO8* encodes a transcriptional activator, acting together with the transcription factor Mss11p in the induction of *FLO* gene expression [50]. The expression of flocculin encoding genes is further repressed by the transcription factor Sfl1. Tpk2, a subunit of protein kinase A, activates Flo8 and inhibits Sfl1 through phosphorylation, consequently activating *FLO11* expression [51-53]. Different phenotypes of flocculent yeasts can be distinguished based on the inhibition of different sugars. Flo1 type yeasts, whose flocculation phenotypes are encoded by the expression of *FLO1*, *FLO2*, *FLO4*, *FLO5*, *FLO9* and/ or *FLO10*, are not

showing flocculation in the presence of mannose [26, 54]. NewFlo type strains, the most frequently occurring phenotypes in *S. pastorianus* strains, however, do not flocculate in the presence of glucose, maltose, sucrose and/ or mannose. NewFlo phenotypes are characteristic for the expression of *LgFLO*, *FLONL* and *FLONS* genes [49, 54]. The less common M1 flocculation type occurs in the presence of ethanol via a protein-protein interaction; it is however not known which genes contribute to this phenotype [26, 49]. *FLO11*, which is the only *FLO* gene not located in a sub-telomeric region, presents an exception in the *FLO* gene family and is involved in filamentous growth [49].

Maturation and conditioning of the beer

When, at the end of the fermentation, most sugars and assimilable nitrogen are depleted, yeast cells stop proliferating but stay metabolically active in the G_0 phase of the cell cycle, in which they remain until the sequential fermentation is started [37, 41, 55]. In lager fermentations, the beer is kept in contact with the yeast cells for multiple days and at low temperature (lagering) to promote assimilation and reduction of vicinal diketones, which are off flavours in lager beers [56]. As by-products of valine and isoleucine biosynthesis, diacetyl (2,3-butanedione) and 2,3-pentanedione, two vicinal diketones that are responsible for a butter-like off-flavour, are formed during beer fermentation. For diacetyl production, first, α-acetolactate is produced from pyruvate in a reaction catalysed by α-acetohydroxyacid synthase (Ilv2p and regulatory subunit Ilv6p). For valine synthesis, acetolactate is, first reduced to 2,3-dihydroxy-isovalerate by Ilv4p and Ilv5p, then dehydrated by Ilv3p and subsequently converted to valine by the addition of a NH_2 group through the Bat1p and Bat2p transaminases [56-58]. Alternatively, α-acetolactate can diffuse out of the cells, after which extracellular, spontaneous oxidative decarboxylation yields diacetyl [58]. Formation of another vicinal diketone, 2,3-pentanedione, is catalysed by the same enzymes, starting with an additional deamination of threonine through Ilv1p, yielding 2-ketobutyrate which is then converted together with a molecule of pyruvate by Ilv2p/Ilv6p. At the end of the fermentation process, during maturation, yeast slowly converts these vicinal diketones, resulting in the formation of diols, which have a much higher flavour threshold (approx. 4500 ppm for 2,3-butanediol compared to < 1 ppm for diacetyl) [19, 56].

At the end of the (primary) fermentation, after maturation, the beer is cooled and conditioned. Filtration leads to a clear, bright and stable beer which is then packaged in bottles, cans or kegs of larger volume. Since the produced beer contains less carbon dioxide than desired, additional carbonation via addition of CO_2 (or via a natural carbonation through a secondary bottle fermentation in unfiltered beers) is applied. The majority of commercial beers are filtered and carbonated with CO_2 since this practice ensures the most stable product. This CO_2 can be derived from the main fermentation, where CO_2 is formed as by-product. Excess CO_2 from the brewing process is initially recovered in order to avoid the accumulation of the toxic gas in beer production facilities.

CO_2 recovery systems can capture, clean and store the gas produced during fermentation so that it can be re-used for carbonation. In this way costs of carbonation, as well as CO_2 emissions to the atmosphere, can be reduced [59, 60]. Captured CO_2 can be used, next to beverage and food processing, as feedstock for the production of fuels and chemicals, for the cultivation of algae, to enhance oil recovery and also for water treatment [61].

Excess yeast from brewing fermentations has a high nutritional value used and is used to produce nutritional supplements, flavour enhancers, spreads like Vegemite, or applied as an animal feed ingredient.

Brewing yeasts

Commonly used brewing yeasts belong to the *Saccharomyces sensu stricto* group (see Figure 3). Next to the *Saccharomyces* species *S. cerevisiae* and *S. eubayanus*, especially hybrids such as *S. cerevisiae* x *S. kudriavzevii*, *S. bayanus* or *S. pastorianus* are of high relevance for industrial fermentation. In the surrounding of beer, wine, and cider fermentation, exchange of genetic material between different *Saccharomyces* species frequently happens. This way, for example, *S. bayanus* (initially defined as a hybrid between *S. eubayanus* and *S. uvarum*, [90, 339]) strains were found that contained introgressions of *S. cerevisiae* [74, 298, 338]. Likewise, hybrids are found between two or more species of the genus *Saccharomyces* that are associated with human activities [289, 340, 341, 342].

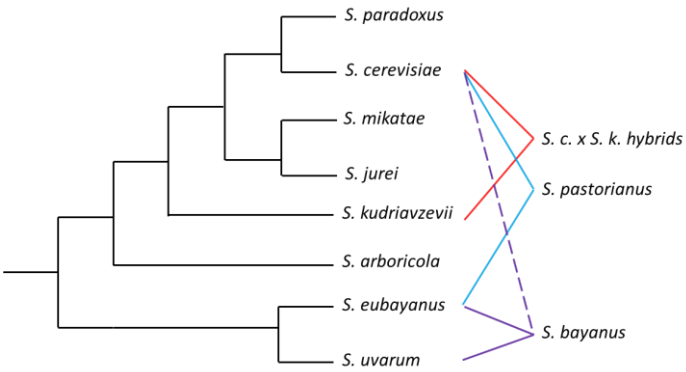

Figure 3: Schematic phylogenetic representation of the *Saccharomyces sensu stricto* group and interspecies hybrids. *S. c.* x *S. k* hybrids are used for wine and cider fermentation, *S. c.* x *S. e.* = *S. pastorianus* hybrids for beer production. *S. bayanus* hybrids are reported containing either genetic material of two (*S. e.* x *S. u.*) or three (additionally *S. c.*) species (*S. c.* introgressions represented by the dotted line). Tree combines findings from different studies and is adapted from [62-65, 74, 298, 338].

Based on their products, yeast strains used to produce beer can be assigned to one of two main groups: ale and lager yeasts. Traditionally, the difference between the two groups was defined according to their behaviour during fermentation. While ale yeasts tend to rise to the top of the fermentation vessel (top fermenting), lager yeasts flocculate and accumulate at the bottom of the tank (bottom fermenting) at the end of the fermentation. Further distinctions can be based on different optimal fermentation

temperatures and on the production of diacetyl. Lager strains generate higher amounts of diacetyl due to a slower valine uptake from the medium and thus a higher flux through metabolic routes towards valine [56] but also show a higher amount of diacetyl reductases which enable faster breakdown of the compound [66]. Another difference lies in the ability to metabolize the disaccharide melibiose. Ale yeast strains cannot ferment melibiose due to the absence of *MEL* genes and a consequent lack of melibiase [67] [68]. *S. cerevisiae* ale yeast strains, commonly able to ferment maltose and maltotriose, are used in ale fermentation processes at 15 – 25 °C [69-71]. It is suggested that today's frequently used ale yeast strains are derived from only a few ancestral strains, which were then adapted during a prolonged domestication process that resulted in improved sugar consumption and stress tolerance. *S. cerevisiae* strains isolated from man-made environments show much higher genome similarity than strains isolated from natural environments [70]. Two subpopulations of ale strains can be distinguished. Ale yeasts commonly used in the US, Britain, Belgium and Germany (ale Group A) contain a functional maltotriose transporter gene *ScAGT1* (at the *MAL1* locus) whereas in another group of ale yeasts, the *MAL11* allele is present on that locus [70]. In a second study, an additional gene, *MTT1*, encoding a maltotriose transporter, was identified in *S. cerevisiae* brewing yeast, which was however only found to be present in one of two ale strains tested (isolated in a British brewery) [71]. The evolution of specific maltotriose transporters seems to be the main reason for the higher maltotriose fermentation capacity of this Group A of ale strains compared to the second group [70].

Saccharomyces pastorianus lager yeasts have probably been in use since the 15th century in low temperature (5 – 15 °C) bottom fermentation processes [1]. *S. pastorianus* yeasts are allopolyploid interspecies hybrids between *S. cerevisiae* and the cold tolerant species *Saccharomyces eubayanus* [72-75], which, in contrast to *S. cerevisiae*, have not been isolated from nature. All currently available evidence suggests that, instead, *S. pastorianus* hybrids are the result of a long domestication and selection process under lager brewing conditions, which aligned their genomes and physiology with the low temperature fermentation and maturation process.

Two major *S. pastorianus* groups exist, which are referred to as the Saaz and Frohberg groups (also called Group 1 and Group 2, respectively). Initially, these groups were thought to be derived from independent hybridization events [74, 76]. The identification of identical interchromosomal translocations shared by yeasts from both groups, however, suggests a common hybrid ancestor [77, 78]. Two possible explanations have been proposed for the distinct ploidies found in Group 1 and 2 strains. A first explanation, developed from ploidy and phylogenetic analysis comparing single nucleotide variants (SNVs) against reference genomes, is based on the assumption that the original hybridization event involved a diploid *S. eubayanus* and a haploid *S. cerevisiae* ale strain, giving rise to the allo-triploid strains of Group 1 (Saaz type). Following this explanation, this hybrid later hybridized again with a haploid *S. cerevisiae* strain, giving rise to strains of Group 2 (Frohberg type) which are allo-tetraploids [77]. The second

explanation assumes that the initial hybrid was of the Frohberg-type and arose from a hybridization of two diploid parents. Still according to the second explanation, subsequent loss of chromosomes, especially in the *S. cerevisiae* subgenome then led to the current Saaz strain lineage. In both scenarios, strains of Group 2 experienced tremendous loss of heterozygosity [78]. After de novo whole genome sequencing of several *S. pastorianus* strains, sequential rounds of hybridization between a diploid *S. eubayanus* and two different haploid *S. cerevisiae* strains have been suggested. Those sequencing results showing Group 2 yeasts to contain two *S. cerevisiae* subgenomes are thus supporting the above described first explanation [78, 79].

The Saaz and Frohberg groups of *S. pastorianus* strains cannot only be distinguished based on genotype, but also through unique phenotypic characteristics, which can be traced back to the relative contributions of the two parental genomes to the hybrid lager yeast genomes. Frohberg strains generally show a faster fermentation and are capable of consuming maltotriose while Saaz strains are more cold tolerant and flocculent than strains of Group 2. In a physiological study of Saaz and Frohberg strains, the tested Saaz strains showed faster growth and a higher ethanol yield at 10 °C whereas at 22 °C the opposite was the case. The tested Frohberg strains fermented 72-84 % of wort maltotriose at 22 °C and only 54 – 65 % at 10 °C. In these studies, Saaz strains did not take up maltotriose [80, 81]. Further, Saaz strains show a lower viability at the end of the fermentation, along with a higher incidence of respiratory-deficient 'petite mutants' than found in corresponding cultures of Frohberg strains [82]. Several studies showed a distinct aroma profile for the two types of lager brewing strains, with Frohberg strains producing higher amounts of isoamyl acetate, isoamyl alcohol and ethyl acetate but lower concentrations of acetaldehyde [35, 80, 82]. In other studies, more variable phenotypes were reported for Group 1 strains. A subgroup of Saaz strains was reported to show a comparable fermentation performance to Frohberg strains regarding maltotriose consumption, with two related Saaz strains (CBS1513 and a variant) showing a higher uptake activity for maltotriose than for maltose and a higher number of *MAL* loci than other strains of both groups [71]. Performance of *S. pastorianus* strains thus is not only group dependent, but, at least for Saaz type strains, also strain dependent. Lager brewing strains of both groups have evolved under the harsh conditions of lager beer brewing for centuries, which led to an extensive genome reorganization (including partial loss of heterozygosity, chromosomal rearrangements and chromosomal copy number variation) [82-84].

The complex genome structure of lager brewing strains leads to challenges when it comes to sequencing of (yeast) hybrid genomes. Initially, the hybrid nature of lager yeast genomes was shown by hybridization studies [72, 85, 86] or by comparative proteomics [87, 88]. Single fragments of *S. pastorianus* genomes were sequenced using Sanger sequencing combined with PCR and restriction fragment length polymorphism (RFLP) analysis [89, 90]. Whole genome sequences of lager brewing yeasts have been obtained by short-read sequencing methods such Sanger sequencing and Illumina

technology [78, 83, 91, 92] and chromosomal copy number (CCN) variations were analysed on the basis of those sequencing reads [93]. Extensive variation in CCN can be found among lager brewing strains and has been linked to industrially relevant phenotypes [94]. In Weihenstephan WS34/70 isolates for example, a higher diacetyl production peak was correlated with an increased copy number of chromosomes harbouring genes involved in valine synthesis [84]. The aneuploidy of the lager brewing yeast genomes (Figure 4) represents a challenge for identification of heterozygous alleles and single-nucleotide variations that can quite easily be detected in euploid or heterozygous diploids [95]. Furthermore, fragmented genome assemblies are often obtained with gaps in repetitive and subtelomeric regions that often harbour genetic information for traits that are relevant for industrial applications [96, 97]. By improving those assemblies, studies of allelic variations and haplotype reconstruction as well as (near) complete reconstruction of aneuploid hybrid genomes will benefit from new long-read sequencing technologies (e.g. the Pacific Biosciences and Oxford Nanopore DNA sequencing platforms) [98-100]. Long-read sequencing resulted in improved genome assemblies of *S. cerevisiae*, providing additional sequence information from reads spanning repetitive and subtelomeric regions as well as insertions of transposable elements and other structural variations [101, 102]. Long-read sequencing can also be used to detect aneuploidy in clinically relevant settings [103, 104] and to reconstruct haplotypes, thereby increasing understanding of the contribution of structural variation to genetic diseases [105, 106].

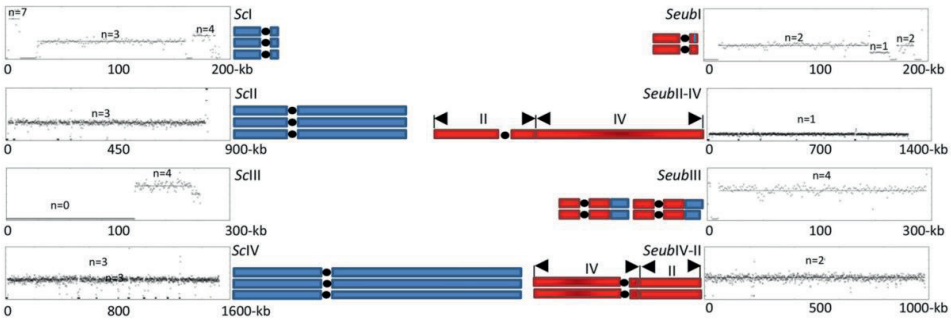

Figure 4: Detail of chromosomal copy number and structure of *S. pastorianus* CBS1483 (CHR. I – IV) adapted from [84]. Blue fragments represent *S. cerevisiae* subgenome parts, red blocks *S. eubayanus* ones.

For the identification of the original donor of the non-cerevisiae subgenome part of *S. pastorianus*, the relatively recent discovery and genome sequencing of *S. eubayanus* [74] were crucial. *S. eubayanus*, the "mother of lager yeasts", as it has been called in an advertisement by HEINEKEN (https://www.heineken.com/ie/H41/Home, Figure 5) was first isolated from fruiting bodies of the fungus *Cyttaria hariotti* growing on *Nothophagus* trees in Patagonia (Argentina) and identified as the non-cerevisiae parent of *S.*

pastorianus lager brewing hybrids [74, 75]. More *S. eubayanus* strains have subsequently been isolated from nature in North America [107], Asia [108] and Oceania [109]. The closest sequence match with the *S. eubayanus* subgenome of *S. pastorianus* was found in an isolate from the Tibetan Plateau (99.82% identity in comparison to 99.56% identity for the original Patagonian isolate) [108]. Based on multi-locus and whole-genome sequencing, *S. eubayanus* strains isolated to date were classified into several lineages. A so-called Holarctic/PB group contains strains isolated in Tibet and North Carolina as well as a *S. eubayanus* lager yeast subgenome (maximum genetic distance determined based on whole genome sequencing is 0.3 %). Also included in this group are strains isolated from Patagonia (lineage PB), which show 0.56 % genetic distance to the first mentioned isolates. A second lineage from Patagonia (lineage PA) is seen as a separate group with a genetic distance of 1.08 % to the Holarctic/PB group. Lineages from Sichuan and West China are most distinct from the other groups (7.4 % distance to the Holarctic group and about 6 % distance to each other) [110]. The Patagonian *S. eubayanus* groups PA and PB have been further divided into subgroups (two groups for PA and three for PB respectively) which reveal a correlation between geographic and genetic distance as well as a link between tree species and predominant yeast species isolated from the former ones [111].

Figure 5: H41, a lager type beer produced by HEINEKEN with a *S. eubayanus* yeast strain isolated in Patagonia.

The availability of multiple isolates from different geographical locations, as well as their genome sequences, stimulated research into the physiology and genetics of *S. eubayanus* strains as well as the generation of new laboratory *S. cerevisiae* x *S. eubayanus* hybrids [34, 99, 112-115]. Studied hybrids have been shown to perform better than both parents regarding maltotriose consumption, cold tolerance, and flocculation behaviour [34, 113, 115-117]. A series of rare mating and spore dissection experiments yielded a hybrid of *S. eubayanus* and two *S. cerevisiae* strains that combined features of all three parents (cold tolerance, maltotriose fermentation and no production of phenolic off flavours) [116]. Studies into newly generated interspecies *Saccharomyces* hybrids not only help to increase understanding of the genetic basis for brewing performance, but also of the evolutionary history of those domesticated hybrids. Further, the construction of novel *S. cerevisiae* x *S. eubayanus* and other interspecies *Saccharomyces* hybrids holds great potential for an increase in the diversity of brewing strains that can be used in industrial applications. A set of hybrids constructed via spore-to-spore mating of *S. cerevisiae* and *S. eubayanus* yeasts yielded strains with a broader temperature tolerance and aroma spectrum. A selected isolate displayed, in addition to an increased production of aromatic compounds, a higher fermentative capacity

compared to its parent strains and a *S. pastorianus* reference in pilot scale experiments [35]. Hybrids constructed with selected Pof$^-$ *S. eubayanus* variants derived from UV-mutagenesis exhibited elimination of the lager-brewing off flavour 4-vinyl guaiacol (4VG) [118]. Interspecies *S. cerevisiae* x *S. eubayanus* hybrids further showed beneficial phenotypes for cider fermentation combining fermentation at cold temperatures with the absence of sulphurous off- flavour production [114].

Maltose and maltotriose utilization by *Saccharomyces* yeasts

While glucose can enter the cell via facilitated diffusion, maltose and maltotriose, the two most abundant sugars in wort, are taken up by *Saccharomyces* yeasts through proton symporters. As a consequence, along with every α-glucoside molecule, a proton is taken up into the cytosol which has to be exported by an ATPase at the cost of one ATP per H^+ [119]. Inside the cytosol, maltose and maltotriose are hydrolysed into α-D-glucose molecules by maltase enzymes acting on α-1,4 bonds, after which glucose enters central carbon metabolism [120, 121]. The transport of α-glucosides across the cell membrane is generally assumed to be an important rate-controlling step in the utilization of maltose and maltotriose [120, 122, 123].

Genes involved in maltose metabolism of brewing related yeasts, first described in *S. cerevisiae*, are clustered on subtelomeric located *MAL* loci which each contain three genes that encode an α-glucoside transporter, an α-glucosidase and a transcriptional activator protein (*MALx1*, *MALx2*, and *MALx3*, respectively, *x* indicating the locus) [124-126]. *S. cerevisiae* and *S. pastorianus* strains vary in their number of *MAL* loci as well as in their copy number and completeness [69, 127, 128]. In *S. pastorianus*, *MAL* loci located on different chromosomes belonging to the *S. cerevisiae* subgenome have been identified, which show a high similarity to each other (up to 99 %, [129]), suggesting a shared evolutionary origin [130]. Duplication of subtelomeric genes, gene families and/or whole chromosomes in brewing yeasts facilitated the evolution of genes encoding maltose and maltotriose transporters, which, after their amplification, could acquire new or evolve specialized functions, resulting in transporters with diverse substrate spectra and affinities [97]. Several families of α-glucoside transporters with different properties are known in brewing related yeasts, including Malx1-like, Agt1, Mph2/Mph3 and Mtt1/Mty1-like transporters [75, 83, 131-134].

Malx1 transporters, even though displaying high similarity to each other, have been shown to exhibit different substrate specificities. Mal61 was, in early studies, describes as a maltose (and turanose) transporter [135, 136]. Maltose and maltotriose uptake was then detected for Mal31 and Mal61 [137]. Mal31 and Mal61 were suggested as potential maltotriose transporters [129]. However, those results were later questioned due to possible contaminations of the sugars used in the uptake assays [132]. Both Mal31

and Mal61 were confirmed as maltose but not maltotriose transporters in several studies [69, 97, 129, 131, 133] and found to be highly expressed in lager brewing strains grown on maltose [138]. Mal11, Mal21, and Mal41, in contrary, showed no uptake activity towards maltose nor were those Malx1 transporters able to transport maltotriose [69, 129, 139, 140].

AGT1, often seen as allele of *MAL11* and encoding a transporter sharing 57 % identity with the latter one and other Malx1 proteins, can be found at the *MAL1* locus [129, 136, 139, 141, 142]. Agt1 transporters were shown to transport both maltose and maltotriose and further have a broad substrate spectrum (including maltose, isomaltose, maltotriose, turanose, sucrose, trehalose, α-methylglucoside, palatinose and melezitose) [97, 122, 129, 139-141, 143]. While *S. cerevisiae* strains can contain either *MAL11* or *AGT1* [144], a vast majority of ale strains contain *AGT1* at the *MAL1* locus [70]. Most *S. pastorianus* lager brewing strains contain *AGT1* genes [69, 128, 131]. Those genes are present on the subgenomes of both parents *S. cerevisiae* and *S. eubayanus*. The *S. cerevisiae AGT1* of all studied lager strains contains a point mutation leading to the presence of a premature stop codon and resulting in a truncated and thus non-functional Agt1 protein [138, 142]. The *AGT1* gene found on *S. eubayanus* chromosome XV-VIII in *S. pastorianus* strains encodes, in contrary to the earlier described one, a functional α-glucoside transporter [71, 83, 134, 145]. *AGT1* was not found in the genome of *S. eubayanus* type strains CBS12357 [75] but *S. pastorianus* contigs showed a near perfect identity to sequences from Holarctic *S. eubayanus* isolates [34, 71, 108].

S. pastorianus strains contain several copies of a further maltose and maltotriose transporter, Mtt1 (also called Mty1) that shares 90% identity with the Malx1 group [134], and is present in most lager brewing yeasts but only found in very few *S. cerevisiae* ale strains [131, 132, 146]. Two versions of *MTT1* exist, with lengths of 2.4 kb and a 2.7 kb, each consisting of a 1,848 bp open reading frame and a promoter region. The longer version is nearly identical to the short one except for an insertion of two repeats in the promoter region of the gene leading to an increased distance between an activator binding site and the open reading frame (ORF). This distance is proposed to be the reason for the loss of function of the longer version of this transporter gene [133]. Mtt1 was shown to display a higher affinity towards maltotriose than towards maltose [71, 132], especially at low temperatures [146].

MPH2 and *MPH3* are homologous genes found in *S. cerevisiae*. *MPH3* has a single nucleotide insertion close to the end of the open reading frame (ORF) resulting in a premature stop codon and a slightly shorter protein than encoded by *MPH2* [147]. Mph2/Mph3 transporters do not seem to play a major role in maltose and maltotriose uptake in brewing yeasts. Initially described as putative maltose and maltotriose transporters (with K_m values of 4.4 and 7.2 mmol L^{-1} for maltose and maltotriose respectively) [147], those results were questioned due to possible contamination in [^{14}C]-maltotriose uptake assays [132]. Later studies showed negligible expression levels of

Mph2/3 transporters in brewing yeasts during growth on maltose [138]. The two homologous transporters were suggested to only transport turanose [97].

In *S. eubayanus* CBS12357, four putative α-glucoside transporters were identified (Malt1 – Malt4) [75], which are characterized in Chapter 2 of this thesis [99]. All genes tested encode functional maltose (but not maltotriose, as also suggested by [71]) transporters when separately expressed in a maltose negative background. In the *S. eubayanus* wildtype, however, *MALT2* and *MALT4*, which share 99.7% identity, are responsible for maltose uptake (Chapter 2).

The performance of individual transporters and their contribution to maltose and maltotriose consumption is well studied in *S. cerevisiae*, but still remains partly unclear in the aneuploid lager yeast hybrid *S. pastorianus* and the recently discovered *S. eubayanus*. This is due to their location in dynamic subtelomeric regions [97], their high homology and copy number in especially *S. pastorianus* lager brewing strains [133], the poor genetic accessibility of *S. pastorianus* strains [148] and lack of consistency in studies on maltose and maltotriose transport in *S. pastorianus* and *S. eubayanus* strains.

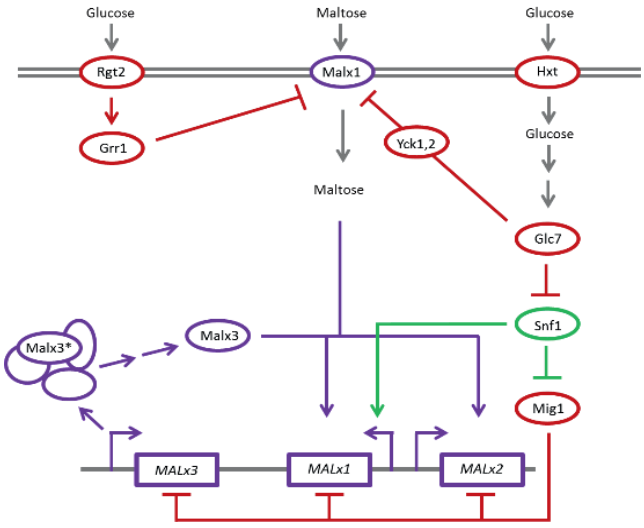

Figure 6: Regulation of a canonical *MAL* locus in *Saccharomyces*. Simplified scheme of signalling pathways. Expression of *MAL* locus is induced in the presence of maltose (purple) and absence of glucose (green) and repressed in the presence of glucose (red). In the presence of maltose, Malx3 is released from a stabilizing complex with Hsp70, Hsp80 and Sti1 and can activate *MALx1* and *MALx2* expression. Snf1, de-repressed in the absence of glucose, is additionally required for the activation of *MALx1*. Snf1 further represses Mig1 and thus reverses repression of *MALx1*, *MALx2*, and *MALx3* expression (through Mig1). In the presence of high extracellular levels of glucose, Malx1 itself is inactivated and degraded via glucose sensing and signalling pathways. Figure adapted from [149, 150].

Maltose and maltotriose uptake is regulated by the presence of these oligosaccharides (Figure 6). In particular, their presence induces expression of the genes encoding transport proteins and maltases via the transcriptional activator Malx3 acting on a divergent promoter between *MALx1* and *MALx2*. Once translated and in the absence of maltose, Malx3 forms a stable intermediate complex with the chaperons Hsp70, Hsp90, and Sti1 to ensure proper folding. When maltose is present inside the cell, Malx3 is cleaved off and can bind to specific activation sites on the *MALx1*/ *MALx2* promoter [150-152]. A higher

number of Malx3 binding sites was shown to increase gene expression [153] and an insufficient number of binding sites could therefore prevent sufficient activation of *MALx1* and *MALx2* expression [99]. For activating the expression of *MALx1*, a further element, the protein kinase Snf1, is required [154]. In contrast to maltose, glucose represses maltose and maltotriose assimilation via multiple processes [149]. In the presence of high levels of extracellular glucose, the glucose sensor Rgt2 signals Grr1, which triggers proteolysis of Malx1 [155]. Similar to Grr1, the expression of a gene pair, *YCK1* and *YCK2*, which is activated during glucose utilisation, causes inactivation and degradation of Malx1 proteins [156]. The expression of *MALx1*, *MALx2*, and *MALx3* is further repressed by the protein Mig1 which is on the other hand repressed by Snf1 [157]. During glucose utilisation, *SNF1* expression is repressed, thereby allowing Mig1 to repress expression of the three *MAL* genes and preventing activation of *MALx1* expression via Snf1. In addition to glucose repression at the level of transcription and catabolite-induced protein degradation, the presence of glucose also leads to post-translational modification and inactivation of transport proteins via phosphorylation or ubiquitination [149, 158]. The described impacts of glucose on maltose transport ultimately result in a sequential fermentation of the wort sugars, with maltose and maltotriose only taken up once the majority of the glucose has been consumed [159, 160].

Complementation of incomplete loci and trans-regulation across hybrid genomes is possible for *MAL* genes in brewing yeasts [161, 162]. Malx3 activators from both *S. cerevisiae* and *S. eubayanus* were shown to activate expression of *MAL* loci of the respective other yeast (shown by analyzing transcript levels of transporter genes) [162].

Yeast strain improvement

Increasing tolerance of brewing yeasts to the stress factors that they are exposed to during beer production (see section 2), prevention of incomplete or inefficient fermentation and a desire to develop new products all provide powerful incentives for yeast strain improvement. Different approaches can be followed to improve industrial yeast strains in order to, for example, increase their productivity and robustness or to develop new aroma profiles in fermented beverages. These approaches can be classified into four main categories: classical breeding and hybridization, random mutagenesis, targeted genetic modification and directed laboratory evolution [163].

Darwin was already aware of the evolutionary advantage of hybrids which exhibit combined traits from both parents and he stressed advantages of (classical) breeding and domestication that would result species with improved characteristics [164, 165]. Hybridization of yeasts, using direct spore-to-spore mating, rare mating or mass mating can generate increased diversity and new combinations of desired traits. Species within the genus *Saccharomyces* have a similar genome, life cycle and mating system and are able to mate with each other [166]. The phenomenon of inter- and intra-species

mating not only occurs in nature (compare Figure 3) and domesticated environments (beer and wine fermentations for example), but can also be used in the laboratory to construct new hybrids for fundamental research and industrial brewing purposes (e.g. [34, 112, 167]).

Genome-wide random mutagenesis, for example induced by treatment with ethane methylsulfonate (EMS) or UV light, can be used to create pools of cells that carry random mutations. These mutagenized populations can then be selected for a specific trait such as increased ethanol tolerance or increased tolerance to high sugar concentrations [168]. Methods relying on random mutagenesis require screening of numerous clones to acquire a strain that inhibits the desired improved phenotype. For application in food products and beverages, it is relevant to note that, despite the often extensive genetic changes that occur during such non-targeted mutagenesis, the resulting strains are considered 'non-GMO' [169].

Targeted genetic engineering, on the other hand, can introduce, delete or alter specific genes known to be responsible for relevant phenotypes in a controlled manner, with minimal unintended 'off target' effects and with strongly reduced screening efforts. Due to their complex genomes, *S. pastorianus* lager brewing yeasts were for a long time difficult to access with standard yeast genetic engineering approaches [163]. This situation is now rapidly changing as a result of to the development of CRISPR tools for these hybrid yeasts [170]. CRISPR-Cas9 mediated gene editing in *S. pastorianus* brewing yeast has been successfully demonstrated using ribozyme flanked gRNA [171, 172]. Genetically engineered strains of *S. cerevisiae* are commonly used in the biotechnological production of transport fuels and pharmaceuticals [173]. In contrast, the potential of genetic engineering for introducing or improving specific traits for industrial brewing purposes has not been exploited because of a lack of acceptance by consumers [174]. While CRISPR-Cas9-mediated allele change helped identifying alleles that can be applied for developing brewing yeast strains that yield improved flavour characteristics to beer [175], genetically engineered strains were not used for industrial brewing purposes in that study. Already in the 1990s, a brewing yeast that had been genetically engineered (via 2 micron plasmid based introduction of a heterologous *DEX1* gene) to utilize dextrins through producing extracellular amyloglucosidase and, thereby, convert more wort carbohydrates to ethanol, was approved by the British government for use in commercial brewing [176, 177]. This early study indicates that regulatory requirements do not necessarily preclude market introduction of beer made with genetically engineered yeast. This yeast, however, did not lead to commercial success. In 2015, an *Arabidopsis thaliana Δpsbs* (a protein involved in photosynthetic light harvesting) mutant engineered using a CRISPR-Cas9 based approach was confirmed by the Swedish government to not fall under the European GMO definition (EU directive 2001/18/EC, [169]), because it does not contain foreign DNA. After this case study, in 2016, cabbage was grown from genetically edited *Brassica* seeds and the harvested vegetables were prepared as "Tagliatelle with CRISPRY fried vegetables" [178]. In the same year, the mushroom *Agaricus bisporus*,

engineered for reduced browning by deleting six genes using CRISPR-Cas9, received a green light from the US government to be cultivated and marketed without further oversight [179]. These decisions were thought by many to be the first examples of widespread introduction of other CRISPR-Cas9 engineered food crops in the near future. In the European Union, this development experienced a major setback through a recent decision made by the Court of Justice of the European Union on July 25th, 2018 (see https://curia.europa.eu/jcms/upload/docs/application/pdf/2018-07/cp180111en.pdf) which states that crops engineered by means of CRISPR-Cas9 or similar techniques, even when they do not contain foreign DNA, are still subject to the 2011 EU GMO directive [180].

Non-traditional breeding methods represent genetic engineering approaches for shuffling and combining yeast genomes of strains which are not able to (efficiently) sporulate [163]. One example is mating by transient HO induction (transforming a homothallic switching (= HO) endonuclease under the regulation of a inducible promoter on a plasmid into cells of also higher ploidy [181]). Using this strategy, marker-free hybrid *Saccharomyces* strains were constructed, yielding new beer and cider yeasts [182]. Furthermore, cytoduction (cell fusion without fusing nuclei [183]) or protoplast fusion are resulting strains with hybridized genomes. Breeding methods can also be used with mixed populations and in combination with a selective pressure to reduce screening effort.

An alternative to GMO methods and classical strain improvement and breeding is evolutionary engineering, also called adaptive laboratory evolution (ALE). Evolutionary engineering is based on selecting for and enriching cells harbouring beneficial mutations for a specific trait via serial batch or continuous fermentation, selection and transfer on solid medium, or serial transfer and cultivation in emulsions [184-186]. The desired outcome of such experiments is that, at the end of long-term cultivation under a carefully designed selective pressure, the population mainly consists of cells with beneficial mutation(s). In serial batch cultivation, strains are selected for an increased specific growth rate, which can be directly coupled to an increased activity of dissimilatory pathways such as, in anaerobic yeast cultures, alcoholic fermentation. For brewing yeasts, this strategy has been successfully applied for improved growth of *S. pastorianus* in high-gravity fermentations, or for reduced ethanol production of *S. cerevisiae* in red wine production [187]. As a side effect, selection for fast sedimenting mutants, which sometimes accumulate in bioreactors during empty-refill cycles, can occur when using serial batch fermentations [188]. A selection for increased affinity for a limiting nutrient can be achieved in nutrient-limited continuous cultivation systems (chemostats). Selection in chemostat cultures has been applied in this thesis for increasing the affinity of *S. pastorianus* for maltotriose uptake [95] (Chapter 4). The stable conditions in a chemostat cultures can select for mutants that perform sub-optimally under dynamic, stressful conditions [189]. As a further strategy, microfluidics can be used to select for beneficial traits that are not correlated with a faster growth rate by enabling

compartmented evolution in emulsions and subsequently applying droplet sorting techniques to identify mutants based on optical density (to select for a high yield) or fluorescence for example [184].

The analysis of evolved phenotypes and the identification of causative mutations is performed via whole genome (re-)sequencing, often followed by reverse engineering of acquired mutations in a non-evolved parental strain in order to assess their significance. The combination of evolutionary engineering with the current rapid advances in sequencing techniques presents a great potential for improving yeast strains used in the production of fermented beverages [190].

Scope of this thesis

The main objective of the research described in this thesis was to develop a better understanding of maltose and maltotriose metabolism in brewing-related *Saccharomyces* yeasts. To this end, the functionality of individual maltose transporters was studied in the *S. eubayanus* type strain CBS12357, while the physiology of a *S. cerevisiae* x *S. eubayanus* hybrid constructed in the laboratory was investigated to better understand the evolutionary advantage and predominance of *S. pastorianus* lager brewing hybrids in lager beer fermentation processes. Maltotriose fermentation, which is often slow and incomplete during lager brewing, was studied and improved in a *S. pastorianus* lager-brewing strain via directed laboratory evolution.

The utilization by brewing yeasts of maltose and maltotriose, the two most abundant sugars in brewing wort, is an important factor for the efficiency of the beer production process and the quality of the final product. However, knowledge on the contribution of different membrane transporters to the uptake of these sugars is far from complete. To better understand the contribution of both parental genomes to maltose fermentation, **Chapter 2** focussed on obtaining and analysing a near-complete genome assembly of the *S. eubayanus* type strain CBS12357, including a complete resolution of all four *MAL* loci, using Oxford Nanopore Technology MinION long-read sequencing technology. Functional characterization of single transporters in a maltose-negative strain background supported the functionality of all maltose transporters from this *S. eubayanus* strain. Deletion of *MALT2* and *4*, which have a 99.7% identical sequence, in the wildtype strain *S. eubayanus* CBS12357 eliminated growth on maltose. This result enabled the conclusion, which was confirmed by transcriptome analysis, that the transporters encoded by these genes play a major role in maltose uptake by this *S. eubayanus strain*. The genomic and physiological characterization performed in this chapter represents a valuable contribution to the understanding of oligosaccharide transport in *S. eubayanus* and can be used as basis for selecting and improving strains for industrial use.

As discussed above, *S. pastorianus* strains used for lager beer brewing descended from natural *S. cerevisiae* x *S. eubayanus* hybrids, which developed aneuploid genome structures during the process of domestication in lager brewing process. To gain a deeper understanding of factors that contributed to the predominance of lager brewing hybrids in such man-made environment, a novel *S. cerevisiae* x *S. eubayanus* hybrid was constructed in the laboratory as described in **Chapter 3**. In the context of this study, this laboratory hybrid was considered to represent a model for the ancestor of current lager brewing yeasts. The hybrid showed heterosis, which means that its performance favourably compared to either parent for several characteristics, including maximum specific growth rate at low temperature as well as oligosaccharide utilization. This study, performed under laboratory conditions, provides a clear indication on how hybridization may have conferred selective advantages to the early ancestors of *S. pastorianus* in beer fermentation processes.

Regardless of the superior performance of *S. pastorianus* lager brewing hybrids in comparison to their parents, in industrial lager beer fermentations on high gravity wort, the affinity of the yeast for maltotriose is not very high and maltotriose often is incompletely fermented at the end of the process. Complete and timely consumption of all wort sugars is important for an economical process as well as for the stability of the product. Based on this inspiration from industrial application, **Chapter 4** describes an evolutionary engineering strategy in sugar-limited chemostat cultures for improving maltotriose fermentation kinetics, applied to an industrial *S. pastorianus* lager brewing strain. This approach resulted in populations with up to 5-fold lower residual maltotriose concentrations. An evolved isolate exhibited a 4.75-fold higher maltotriose transport capacity than the parental strain in uptake studies with ^{14}C-labelled sugars. The improved characteristics of the evolved *S. pastorianus* were reproduced at industrial pilot scale. Pilot fermentations at 1,000 L scale demonstrated that an evolved strain could produce more beer, with a lower maltotriose content, from the same amount of wort, while retaining a flavour profile that was compatible with specification for lager beer. The results demonstrated the potential of the use of evolutionary engineering to improve brewing yeasts with non-GM methods and to efficiently enhance the performance of industrial strains with complex, aneuploid genomes.

Chapter 2: Structural, physiological and regulatory analysis of maltose transporter genes in *Saccharomyces eubayanus* CBS 12357^T

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Essentially as published in **Frontiers in Microbiology** 2018, 9:1786 (https://doi.org/10.3389/fmicb.2018.01786)

Abstract

Saccharomyces pastorianus lager brewing yeasts are domesticated hybrids of *Saccharomyces cerevisiae* and cold-tolerant *Saccharomyces eubayanus*. To understand the contribution of both parental genomes to maltose metabolism in brewing wort, this study focuses on maltose transport in the *S. eubayanus* type strain CBS 12357^T/FM1318. To obtain complete sequences of the *MAL* loci of this strain, a near-complete genome assembly was generated using the Oxford Nanopore Technology MinION sequencing platform. Except for CHRXII, all sixteen chromosomes were assembled as single contigs. Four loci harboring putative maltose transporter genes (*SeMALT1-4*), located in subtelomeric regions of CHRII, CHRV, CHRXIII, and CHRXVI, were completely resolved. The near-identical loci on CHRV and CHRXVI strongly resembled canonical *S. cerevisiae MAL* loci, while those on CHRII and CHRXIII showed different structures suggestive of gene loss. Overexpression of *SeMALT1-4* in a maltose-transport-deficient *S. cerevisiae* strain restored growth on maltose, but not on maltotriose, indicating maltose-specific transport functionality of all four transporters. Simultaneous CRISPR-Cas9-assisted deletion of only *SeMALT2* and *SeMALT4*, which shared 99.7% sequence identity, eliminated growth of *S. eubayanus* CBS 12357^T on maltose. Transcriptome analysis of *S. eubayanus* CBS12357^T established that *SeMALT1* and *SeMALT3*, are poorly expressed in maltose-grown cultures, while *SeMALT2* and *SeMALT4* were expressed at much higher levels than *SeMALT1* and *SeMALT3*, indicating that only *SeMALT2/4* are responsible for maltose consumption in CBS 12357^T. These results represent a first genomic and physiological characterization of maltose transport in *S. eubayanus* CBS 12357^T and provides a valuable resource for further industrial exploitation of this yeast.

Introduction

Saccharomyces eubayanus was first isolated from Nothofagus trees and stromata of *Cyttaria harioti* in North-Western Patagonia [74]. Strains of *S. eubayanus* have subsequently been also isolated from locations in North America [107], Asia [108], and Oceania [109]. Initial physiological characterization of the Patagonian *S. eubayanus* strain CBS 12357^T revealed that it grows faster than *S. cerevisiae* at temperatures below 10°C [34], shows poor flocculation [112], and consumes maltose but not maltotriose [34, 167].

Isolation and characterization of *S. eubayanus* provided a strong impetus for research on *S. pastorianus* lager brewing yeasts. The hybrid nature of lager yeast genomes was already shown by Southern hybridization [85, 86], RFLP genotyping, Sanger sequencing [89, 90], and comparative proteomics [87, 88]. However, release of the first *S. eubayanus* genome sequence [74] unequivocally established that this cold-tolerant *Saccharomyces* species contributed the non-*cerevisiae* part of *S. pastorianus* genomes [77, 82-84]. Access to this genome sequence and its updates [34, 75] proved invaluable for resolving the complex structure of aneuploid *S. pastorianus* genomes. Moreover, access to *S. eubayanus* strains stimulated vigorous research into de novo generation of hybrids between *S. cerevisiae* and *S. eubayanus* in the laboratory [34, 112-115]. This approach has the potential to increase our understanding of the domestication process of lager brewing strains and, moreover, to strongly increase the genetic and phenotypic variety of lager yeast strains available to the brewing industry. De novo constructed *S. cerevisiae* × *S. eubayanus* hybrids have been demonstrated to combine advantageous brewing-related properties of both parents (cryotolerance, maltotriose utilization, and strong flocculation) and even exhibited best parent heterosis also referred to as hybrid vigor [34, 113, 115-117]. However, generation of new hybrids is, by itself, not sufficient to understand the genetic basis for the exceptional performance of *S. pastorianus* under brewing conditions.

Lager brewing strains of *S. pastorianus* have, over several centuries, been selected for rapid, near-complete fermentation of all-malt brewer's wort fermentable sugars, which typically comprise 60% maltose, 25% maltotriose, and 15% glucose, with trace amounts of fructose [123]. Lager brewing therefore critically depends on the capacity of *S. pastorianus* strains to efficiently take up and ferment the wort α-glucosides maltose and maltotriose. The required maltose fermentation characteristics of *S. pastorianus* strains are conferred by genes originating from each of the parents and from a set that likely arose during its domestication history (e.g., MTT1) [47, 71, 129, 131, 132, 134, 138, 191].

In *S. cerevisiae*, maltose metabolism and the responsible *MAL* genes are well characterized in term of sequence, genetics, regulation and biochemistry. *S. cerevisiae MAL* loci harbor the three key genes essential for maltose utilization, encoding a transcriptional activator (*MALx3*), a maltose permease (*MALx1*) and a maltase (*MAx2*)

[130]. Numbers and identities of *MAL* loci are highly strain dependent, with up to five *MAL* loci (*MAL1*, *2*, *3*, *4*, and *6*) occurring in haploid *S. cerevisiae* genomes. *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 [130, 137, 143, 191-194].

Maltose is transported across the *S. cerevisiae* plasma membrane by maltose-proton symport, mediated by Malx1 transporters [119, 195] and, to a lesser extent, by facilitated diffusion [147]. All *MALx1* genes are highly similar, with the exception of *MAL11* and its allele *AGT1*, whose DNA sequence shows only 57% identity to the other four *MALx1* transporter genes [142, 143]. This sequence difference is accompanied by a difference in substrate range, with Agt1 also being able to transport other α-glucosides, such as trehalose [196], sucrose [197, 198], importantly for brewing applications, maltotriose [129, 142]. The *S. cerevisiae* genome harbors two additional maltose permease genes, *MPH2* and *MPH3*, which are located subtelomerically on CHRIV and X, respectively. Although the transport mechanisms of Mph2 and Mph3 have not been experimentally established, both carriers were initially suggested to transport of a range of substrates including glucose, maltose, maltotriose, α-methylglucoside, and turanose [147]. The role of those transporters for maltose and maltotriose transport in brewing yeasts, however, was questioned (see ref. 132, 138, 97 Chapter 1).

In contrast to the wealth of information on *S. cerevisiae*, knowledge on maltose transport in *S. eubayanus* is limited. The type strain *S. eubayanus* CBS 12357^T grows on maltose, but not on maltotriose [34]. Annotation of its genome sequence revealed four open reading frames sharing similarity with *S. cerevisiae MAL31* (*SeMALT1*; *SeMALT2*, *SeMALT3*, and *SeMALT4*) [75]. The hybrid *S. pastorianus* genome harbors two additional maltose transporter gene variants that were not found in either of the reference parental genomes. The first of these, *MTT1*/*MTY1*, shares 90 and 54% DNA sequence identity with *S. cerevisiae MAL31* and *MAL11*, respectively [131, 132]. The second *S. pastorianus*-specific maltose-transporter gene, *SeAGT1*, shared significant identity with *S. cerevisiae AGT1* (85% *ScAGT1*; [145]). *SeAGT1* was unexpectedly found to be located on the *S. eubayanus* derived CHRVIII-XV, suggesting a *S. eubayanus* origin, despite the absence of similar genes in currently available *S. eubayanus* genome sequences [83, 84] However, genome assembly of an Asian *S. eubayanus* strain (CDFM21L.1; [108]) revealed short (<200 bp) sequences reminiscent of a putative *SeAGT1* gene [34]. Both *MTT1*/*MTY1* and *SeAGT1* were shown to confer low- temperature dependent transport of both maltose and maltotriose [199]. To illustrate the complexity of α-glucoside transport in *S. pastorianus*, the model strain Weihenstephan 34/70 contains all *S. cerevisiae MAL* loci except for *MAL2*, a single *MPH* gene (*MPH2*), as well as all four *S. eubayanus* genes (*MALT1* to *4*) [83, 84] and the two *S. pastorianus*-specific genes *MTT1* and *SeAGT1* [71]. In this *S. pastorianus* background, the *S. cerevisiae* allele of *AGT1* (*MAL11*) carries a nonsense mutation [142].

Hitherto, no study has systematically investigated the functionality of the individual α-glucoside transporters in *S. pastorianus*. In addition to the complexity of maltose metabolism in *S. pastorianus* strains, genetic analysis is complicated by the

limited genetic accessibility of industrial lager brewing yeasts [148]. However, availability of the *S. eubayanus* type strain and of its genome sequence offers an alternative approach to fill existing knowledge gaps on transport of wort sugars. The aim of this study was therefore to investigate the contribution of individual putative maltose-transporter (*SeMALT*) genes in *S. eubayanus* CBS 12357^T. To this end, a new near-complete genome sequence of the strain CBS 12357^T was assembled using Oxford Nanopore Technology's MinION long-read sequencing platform. Subsequently, CRISPR-Cas9 gene editing was used to systematically delete the *MALT* genes in *S. eubayanus*. In a complementary approach, all four *S. eubayanus MALT* open reading frames were cloned and constitutively expressed alongside the *S. cerevisiae MAL12* maltase gene in a *S. cerevisiae* strain lacking all maltose utilization genes (*MALx1*, *MALx2*, and *MALx3*), *MPH2/3*, *SUC2*, and *IMA1-5* genes; [200]. Subsequently, growth of the genetically modified yeast strains was analyzed on different carbon sources. Finally, RNA sequencing was performed on glucose- and maltose-grown cultures to study differential expression of the *S. eubayanus MALT* genes.

Materials and methods

Strains and maintenance

S. eubayanus strain CBS 12357^T (alias FM1318, [74]) was obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands, http://www.westerdijkinstitute.nl/). The *S. cerevisiae* strain IMZ616 [200] was derived from the CEN.PK lineage [101, 201]. All strains used in this study are listed in Table 1. Stock cultures of *S. eubayanus* and *S. cerevisiae* strains were grown in YPD (10g L^{-1} yeast extract, 20g L^{-1} peptone, and 20g L^{-1} glucose) until late exponential phase, complemented with sterile glycerol to a final concentration of 30% (v/v) and stored at −80 °C as 1.5ml aliquots until further use.

Media and cultivation

S. eubayanus batch cultures were grown on synthetic medium (SM) containing 3.0g L^{-1} KH_2PO_4, 5.0g L^{-1} $(NH_4)_2SO_4$, 0.5g L^{-1} $MgSO_4$, 7 H_2O, 1mL L^{-1} trace element solution, and 1mL L^{-1} vitamin solution [202]. The pH was set to 6 with 2M KOH prior to autoclaving at 120°C for 20min. Vitamin solutions [202] were sterilized by filtration and added to the sterile medium. Concentrated sugar solutions were autoclaved at 110°C for 20min and added to the sterile flasks to give a final concentration of 20g L^{-1} carbon source [glucose (SMG), maltose (SMM) or maltotriose (SMMt)]. *S. cerevisiae* batch cultures were grown on SM supplemented with 150mg L^{-1} uracil to compensate for loss of plasmid pUDC156 that carried the *cas9* endonuclease gene and supplemented with 20g L^{-1} carbon source

[glucose (SM_uG), maltose (SM_uM) or maltotriose (SM_uMt)]. All batch cultures were grown in 500mL shake flasks with a working volume of 100mL. The cultures were inoculated at an initial OD_{660nm} of 0.1 and incubated under an air atmosphere and shaken at 200 rpm and at 20°C in a New Brunswick™ Innova44 incubator (Eppendorf Nederland B.V, Nijmegen, The Netherlands).

Selection of the *S. eubayanus* strains transformed with plasmids pUDP062 ($gRNA_{SeMALT1}$), pUDP063 ($gRNA_{SeMALT2}$), and pUDP064 ($gRNA_{SeMALT3}$) was carried out on a modified SMG medium, in which $(NH_4)_2SO_4$ was replaced by 5g.L^{-1} K_2SO_4 and 10mM acetamide ($SM_{Ace}G$) [203]. SM- based solid medium contained 2% Bacto Agar (BD, Franklin Lakes, NJ). Selection of *S. cerevisiae* integration strains was carried out on $SM_{Ace}G$. For plasmid propagation, *E. coli* XL1Blue-derived strains (Agilent Technologies, Santa Clara, CA) were grown in Lysogeny Broth medium (LB, 10g L^{-1} tryptone, 5g L^{-1} yeast extract, 5g L^{-1} NaCl) supplied with 100mg L^{-1} ampicillin.

Table 1: Strains used in this study

Strain	Genotype	Species	Reference
CBS12357^T / FM1318	*MATa/MATα SeMALT1/SeMALT1 SeMALT2/SeMALT2 SeMALT3/SeMALT3 SeMALT4/SeMALT4*	Se[a]	[74]
IMK816	*MATa/MATα SemalT1Δ/SemalT1Δ SeMALT2/SeMALT2 SeMALT3/SeMALT3 SeMALT4/SeMALT4*	Se	This study
IMK817	*MATa/MATα SeMALT1/SeMALT1 SemalT2Δ/SemalT2Δ SeMALT3/SeMALT3 SemalT4Δ/SemalT4Δ*	Se	This study
IMK818	*MATa/MATα SeMALT1/SeMALT1 SeMALT2/SeMALT2 SemalT3Δ/SemalT3Δ SeMALT4/SeMALT4*	Se	This study
IMZ616	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*Spcas9::URA3 CEN6*)	Sc[b]	[200]
IMX1253	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::$ScTEF1_{pr}$-SeMALT1-$ScCYC1_{ter}$::$ScTDH3_{pr}$-ScMAL12- $ScADH1_{ter}$* pUDC156 (*Spcas9::URA3 CEN6*)	Sc	This study
IMX1254	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: $ScTEF1_{pr}$-SeMALT2-$ScCYC1_{ter}$::ScTDH3pr-ScMAL12- $ScADH1_{ter}$* pUDC156 (*Spcas9::URA3 CEN6*)	Sc	This study
IMX1255	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: $ScTEF1_{pr}$-SeMALT3-$ScCYC1_{ter}$::$ScTDH3_{pr}$-ScMAL12- $ScADH1_{ter}$* pUDC156 (*Spcas9::URA3 ARS4 CEN6*)	Sc	This study
IMX1365	*MATa ura3-52 HIS3 LEU2 TRP1 mal1Δ::loxP mal2Δ::loxP mal3Δ::loxP mph2Δ::loxP mph3Δ::loxP suc2Δ::loxP-KanMX-loxP ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: $ScTEF1_{pr}$-ScAGT1-$ScCYC1_{ter}$::$ScTDH3_{pr}$-ScMAL12- $ScADH1_{ter}$* pUDC156 (*Spcas9::URA3 ARS4 CEN6*)	Sc	This study

[a] *S. eubayanus*

[b] *S. cerevisiae*

Plasmid and strain construction

Plasmid construction

Guide-RNA (gRNA) sequences for deletion of *SeMALT1*, *SeMALT2/T4* and *SeMALT3* were designed following the guiding principles recommended in [171]. The DNA sequences encoding these gRNAs were synthesized at GeneArt (Thermo Fisher Scientific, Waltham, MA) and were delivered in pUD631, pUD632, and pUD633 respectively (Table 2). The gRNA spacer sequences (*SeMALT1* 5′ATTCCAAACGACAATAAAGA3′, *SeMALT2/T4* 5′-TACAGGAGAATGGGAGATTT-3′ and *SeMALT3* 5′GTTTTCAAAGCTTGCAGAAG-3′) and the structural gRNA sequence were flanked at their 5′ ends by the Hammerhead ribozyme (HH) and at their 3′ ends by the Hepatitis Delta Virus ribozyme (HDV) [172]. The HH-gRNA-HDV fragment was flanked on both ends with a BsaI site for further cloning [171, 204]. In the next step, the gRNAs were transferred into the pUDP004 plasmid [171], which enables combined expression of the gRNA cassette and of Spcas9^{D147YP411T} [205]. The plasmid pUDP062, expressing gRNA$_{SeMALT1}$ was constructed in a one-pot reaction by digesting pUDP004 and pUD631 using BsaI and ligating with T4 ligase. Similarly, pUDP063, expressing gRNA $_{SeMAT2/T4}$ and Spcas9^{D147YP411T} was assembled from pUDP004 and pUD632. The plasmid pUDP064 expressing gRNA$_{SeMALT3}$ and Spcas9^{D147YP411T} was assembled from pUDP004 and pUD633. Correct assembly of pUDP062-064 was verified by restriction analysis with SspI and PdmI.

The coding regions of *SeMALT1*, *SeMALT2* and *SeMALT3* were amplified from CBS 12357^{T} genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), according to the supplier's instructions with primers pairs 10491/10492, 10632/10633, and 10671/10672 (Table S5), respectively. The coding sequence of *ScAGT1* was amplified from CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), according to the supplier's instructions with primers pairs 9940/9941. Each primer carried a 40 bp extension complementary to the plasmid backbone of p426-TEF-amdS [200, 203], which was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) and primers 7812 and 5921 (Table S5). p426-TEF-amdS is an expression plasmid that harbors the promoter of the translational elongation factor EF-1 alpha (*TEF1*) of *S. cerevisiae*. Each *SeMALT* fragment was assembled with the p426-TEF-amdS backbone fragment using NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA), resulting in plasmids pUD479 (*SeMALT1*), pUD480 (*SeMALT2/T4*), pUD481 (*SeMALT3*), and pUD445 (*ScAGT1*) (Table 2).

Table 2: Plasmids used in this study

Name	Relevant characteristics	Origin
p426-TEF-amdS	ori (ColE1) *bla* 2µ amdSYM *TEF1$_{pr}$-CYC1$_{ter}$*	[203]
pUDP004	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$*-BsaI-BsaI-Sc*CYC1$_{ter}$ AaTEF1$_{pr}$-Spcas9$^{D147Y\ P411T}$-ScPHO5$_{ter}$*	[171]
pUDP052	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$- gRNA$_{SGA1}$*-Sc*CYC1$_{ter}$ AaTEF1$_{pr}$-Spcas9$^{D147Y\ P411T}$-ScPHO5$_{ter}$*	This study
pUDP062	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$- gRNA$_{SeMALT1}$*-Sc*CYC1$_{ter}$ AaTEF1$_{pr}$-Spcas9$^{D147Y\ P411T}$-ScPHO5$_{ter}$*	This study
pUDP063	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$- gRNA$_{SeMALT2/T4}$*-Sc*CYC1$_{ter}$ AaTEF1$_{pr}$-Spcas9$^{D147Y\ P411T}$-ScPHO5$_{ter}$*	This study
pUDP064	ori (ColE1) *bla* panARSopt amdSYM Sc*TDH3$_{pr}$- gRNA$_{SeMALT3}$*-Sc*CYC1$_{ter}$ AaTEF1$_{pr}$-Spcas9$^{D147Y\ P411T}$-ScPHO5$_{ter}$*	This study
pUDE044	ori (ColE1) *bla* 2µ *ScTDH3$_{pr}$-ScMAL12-ScADH1$_{ter}$ URA3*	[197]
pUD479	ori (ColE1) *bla* 2µ amdSYM *ScTEF1$_{pr}$-SeMALT1-ScCYC1$_{ter}$*	This study
pUD480	ori (ColE1) *bla* 2µ amdSYM *ScTEF1$_{pr}$-SeMALT2/4-ScCYC1$_{ter}$*	This study
pUD481	ori (ColE1) *bla* 2µ amdSYM *ScTEF1pr-SeMALT3-ScCYC1ter*	This study
pUD445	ori (ColE1) *bla* 2µ amdSYM *ScTEF1pr-ScAGT1-ScCYC1ter*	This study
pUDR119	ori (ColE1) *bla* 2µ AmdSYM *SNR52*$_{pr}$-gRNA$_{SGA1}$-*SUP4$_{ter}$*	[212]

Strain construction

S. eubayanus IMK816 (*SemalT1Δ*) was constructed by transforming CBS 12357^T by electroporation [171] with 200 ng of pUDP062 and 1 µg of 120 bp repair fragment obtained by mixing an equimolar amount of primers 11850 and 11851 (Table S5) [206] (Figure 1). As control, the same transformation was performed without including the repair DNA fragment. Transformants were selected on SM^{Ace}G plates. Strain IMK817 (*SemalT2Δ SemalT4Δ*) and IMK818 (*SemalT3Δ*) were constructed in the same way. The *SeMALT2/T4* deletion was constructed by co-transforming pUDP063 and a repair DNA fragment formed by primers 11328 and 11329, while the *SeMALT3* deletion involved pUDP064 and a repair DNA formed by primers 11330 and 11331 (Table S5). Deletion of *SeMALT1*, *SeMALT2/T4*, and *SeMALT3* was verified by diagnostic PCR, using primers pairs 11671/11672, 11673/11674, and 11675/11676 (Table S5), respectively (Figure 1C). Prior to storing at −80°C, transformants were successively streaked on SM$_{Ace}$G and YPD plates. The genotype was verified after each plating round with the primer pairs mentioned above.

S. cerevisiae IMZ616 [*mal1Δ mal2Δ mal3Δ mph2Δ mph3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*Spcas9 URA3 ARS4 CEN6*)], which cannot grow on α-glucosides [200] was used as a host to test the functionality of individual *S. eubayanus*

(putative) maltose transporter genes. *S. cerevisiae* IMX1253 was constructed by integrating the *S. cerevisiae* maltase gene *ScMAL12* and the *SeMALT1* transporter gene at the *ScSGA1* locus of strain IMZ616 (Figure 2). The *ScSGA1* gene encodes an intracellular sporulation-specific glucoamylase [207] that is not expressed during vegetative growth [208]. This integration site was shown suitable for expression of single or multiple genes as previously demonstrated in [206, 209-211]. The fragment containing *ScMAL12* was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) from pUDE044 [197] with primers 9596 and 9355, which included a 5′ extension homologous to the upstream region of the *S. cerevisiae SGA1* locus and an extension homologous to the co-transformed transporter fragment, respectively. The DNA fragment carrying the *S. eubayanus SeMALT1* maltose symporter was PCR amplified from pUD479 using primers 9036 and 9039, which included a 5′ extension homologous to the co-transformed transporter fragment and an extension homologous to the downstream region of the *S. cerevisiae SGA1* locus, respectively. To facilitate integration in strain IMZ616, the two PCR fragments were co-transformed with plasmid pUDR119, which expressed a gRNA targeting *ScSGA1* (spacer sequence: 5′-ATTGACCACTGGAATTCTTC-3′) [212] (Figure 2A). The plasmid and repair fragments were transformed using the LiAc protocol [213] and transformed cells were plated on $SM_{Ace}G$. Correct integration was verified by diagnostic PCR with primers pairs 4226/5043 and 942/4224 (Figure 2, Table S5). Strains *S. cerevisiae* IMX1254, IMX1255, and IMX1365 were constructed following the same principle, but instead of using pUD479 to generate the transporter fragment, pUD480 pUD481 and pUD445 were used to PCR amplify *SeMALT2/T4*, *SeMALT3*, and *ScAGT1* respectively. Correct integration was verified by diagnostic PCR with primers pairs 4226/5043 and 942/4224 (Figure 2, Table S5). All PCR-amplified gene sequences were Sanger sequenced (Baseclear, Leiden, The Netherlands).

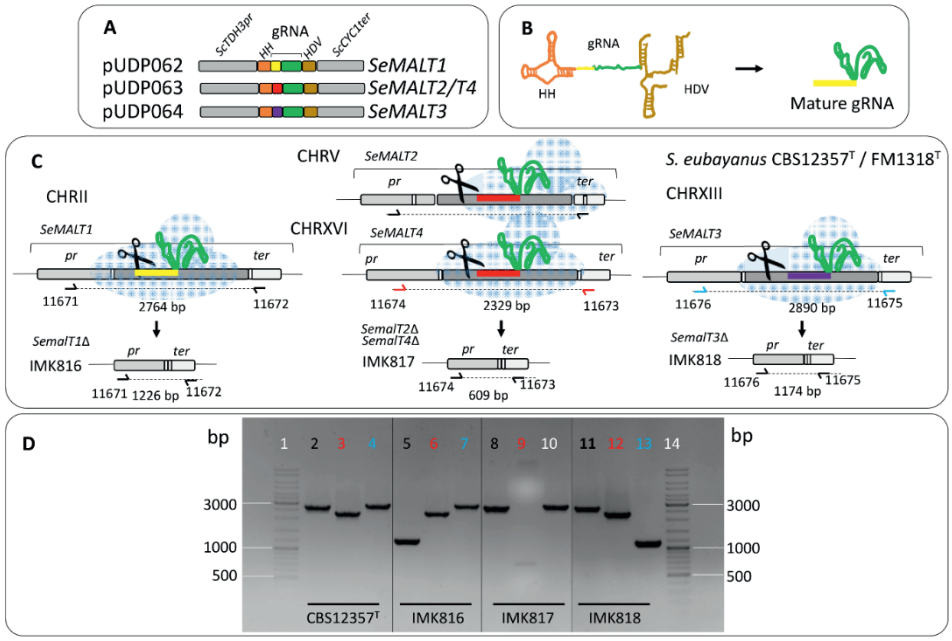

Figure 1: **Deletion of *SeMALT* genes using CRISPR-Cas9-assisted genome editing in *S. eubayanus* CBS 12357^{T}. A** Representation of the gRNA expression cassette in pUDP062, pUDP063 and pUDP064. gRNAs targeting either *SeMALT1*, *SeMALT2/T4* or *SeMALT3* were flanked by a 5' hammerhead ribozyme (HH, orange) and a 3' hepatitis-δ virus ribozyme (HD, bronze). These constructs were expressed from the RNA polymerase II *ScTDH3* promoter and the *ScCYC1* terminator. **B** Upon ribozyme self-cleavage, a mature gRNA comprising the Se*MALT* guiding spacer (yellow) and the constant structural gRNA fragment (green) is released. **C** Schematic representation of Se*MALT* gene editing upon transformation of pUDP062 or pUDP063 or pUDP 064 into *S. eubayanus* CBS 12357^{T}. Primers used for verification of transformants from transformation are indicated together with the size of the expected PCR products. **D** Validation of transformants derived from transformations of *S. eubayanus* CBS 12357^{T} with either pUDP062, pUDP063 or pUDP064 in presence of the corresponding 120 bp repair DNA fragments. Lanes 1 and 14 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). Lanes 2, 5, 8 and 11 fragments amplified with primers 11671 and 11672 black label). Lanes 3, 6, 9, 12 fragments amplified with primers 11674 and 11673 (red label). Lanes 4, 7, 10 and 13 fragments amplified with primers 11676 and 11675 (blue label) from genomic DNA from CBS 12357^{T} (Lanes 2, 3 and 4), from IMK816 (*SemalT1Δ*) (Lanes 5, 6 and 7), from IMK817 (*SemalT2Δ/ SemalT4Δ*) (Lanes 8, 9 and 10) and from IMK 818 (*SemalT3Δ*) (Lanes 11, 12 and 13).

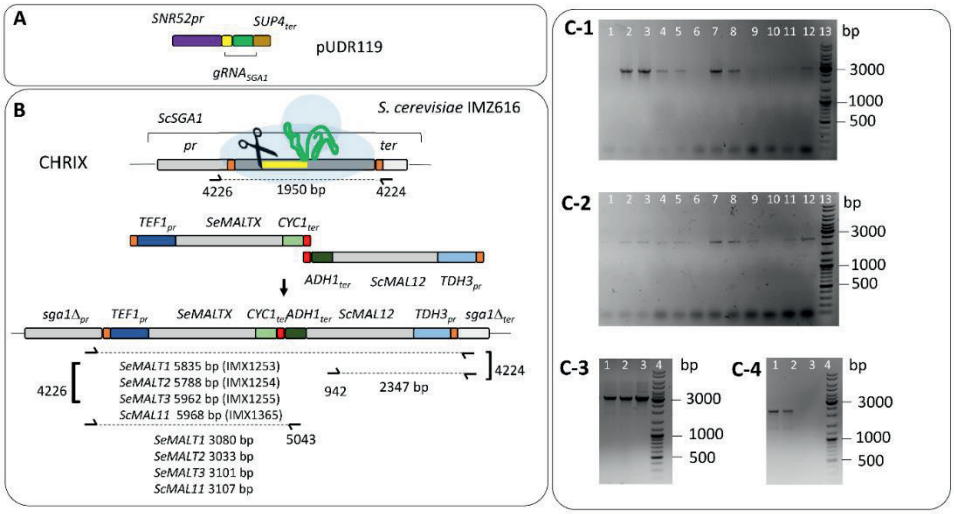

Figure 2: **Integration of *S. eubayanus* CBS 12357^T maltose transporter genes at the *ScSGA1* locus of *S. cerevisiae* IMZ616 (*mal1Δ mal2Δ mal3Δ mph2Δ mph3Δ::suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ Spcas9*) [200]**. **A** Integration at the Sc*SGA1* locus by Cas9-assisted genome editing. The Cas9-targeting gRNA was expressed from pUDR119 [212]. **B** Schematic representation of the integration of *SeMALT* expression cassettes at the *ScSGA1* locus. Upon cleavage, the Cas9-induced double strand break was repaired by the two co-transformed fragments harboring a transporter gene expression cassette and the *S. cerevisiae* maltase gene *MAL12*, respectively. Primers used for verification of transformants from transformation are indicated together with the size of the expected PCR products. Integration of *SeMALT1*, *SeMALT2*, *SeMALT3* or *ScMAL11* resulted in *S. cerevisiae* strains IMX1253, IMX1254, IMX1255 and IMX1365 respectively. **C** Validation of the *S. cerevisiae* IMX1253, IMX1254, IMX1255 and IMX1365. **C-1** Lane 13 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). Lanes 1 to 12 fragments amplified with primers 4226 and 5043. Lanes 1, 2, 3 and 4 fragments amplified from clones transformed with *SeMALT1*. The strain corresponding to lane 3 was renamed IMX1253. Lanes 5, 6, 7 and 8 fragments amplified from clones transformed with *SeMALT2*. The strain corresponding to lane 7 was renamed IMX1254. Lanes 9, 10, 11 and 12 fragments amplified from clones transformed with *SeMALT3*. The strain corresponding to lane 12 was renamed IMX1255. **C-2** Lane 13 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). Lanes 1 to 12 fragments amplified with primers 942 and 4224. Lanes 1, 2, 3 and 4 fragments amplified from clones transformed with *SeMALT1*. Lane 3 corresponds to IMX1253. Lanes 5, 6, 7 and 8 fragments amplified from clones transformed with *SeMALT2*. Lane 7 corresponds to IMX1254. Lanes 9, 10, 11 and 12 fragments amplified from clones transformed with *SeMALT3*. Lane 12 corresponds to IMX1255. **C-3** Lanes 1 to 3 fragments amplified with primers 4226 and 5043. Lanes 1, 2, 3 and 4 fragments amplified from clones transformed with *ScMAL11*. The strain corresponding to lane 3 was renamed IMX1365. Lane 4 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific). **C-4** Lanes 1 to 4 fragments amplified with primers 942 and 4224. Lanes 1, 2, and 3 fragments amplified from clones transformed with *ScMAL11*. Lane 3 corresponds to IMX1365. Lane 4 GeneRuler DNA Ladder Mix (Thermo Fischer Scientific).

Genome sequencing

Illumina sequencing

Genomic DNA from *S. eubayanus* CBS 12357^T was isolated as previously described in [84]. Paired-end sequencing (2-fold 150 bp) was performed on a 350 bp PCR-free insert library using Illumina HiSeq2500 (San Diego, CA) by Novogene (HK) Company Ltd (Hong Kong, China) with a sample size of 3.2 Gbase. Sequence data are available at NCBI under Bioproject accession number PRJNA450912.

MinION sequencing

For Nanopore sequencing, a 1D sequencing library (SQK-LSK108) was prepared according to the manufacturer's recommendation and loaded onto an FLO-MIN106 (R9.4) flow cell, connected to a MinION Mk1B unit (Oxford Nanopore Technology, Oxford, United Kingdom). MinKNOW software (version 1.5.12; Oxford Nanopore Technology) was used for quality control of active pores and for sequencing. Raw files generated by MinKNOW were base called using Albacore (version 1.1.0; Oxford Nanopore Technology). Reads, in fastq format, with a minimum length of 1,000 bp were extracted, yielding 3.26 Gb of sequence with an average read length of 8.07kb. Sequencing data are available at NCBI under Bioproject accession number PRJNA450912.

De novo assembly

De novo assembly of the Oxford Nanopore MinION dataset was performed using Canu (v1.4, setting: genomesize = 12m; [214]). Assembly correctness was assessed using Pilon [215] and further correction "polishing" of sequencing/assembly errors was performed by aligning Illumina reads with BWA [216] using correction of only SNPs and short indels (–fix bases parameter). Genome assembly gene annotation was performed with the MAKER2 annotation pipeline (version 2.31.9) [217] using SNAP (version 2013–11-29) [218] and Augustus (version 3.2.3) [219] as *ab initio* gene predictors. *S. cerevisiae* S288C EST and protein sequences were obtained from SGD (*Saccharomyces* Genome Database, http://www.yeastgenome.org/) and were aligned using BLASTX on the obtained polished sequence assembly (BLAST version 2.2.28+) [220]. Predicted translated protein sequences of the final gene model were aligned to the *S. cerevisiae* S288C protein Swiss-Prot database using BLASTP (http://www.uniprot.org/). Custommade Perl scripts were used to map systematic names to the annotated gene names. Error rates in the nanopore-sequencing data were estimated from the q score (Phred scaled) per read, as calculated by the base caller Albacore (version 1.1.0) (Oxford Nanopore Technology). Average q score was used to calculate the error $P = 10^{q/10}$.

Transcriptome analysis

RNA isolation

S. eubayanus CBS 12357^T was grown in either SMG or SMM until mid-exponential phase (OD_{660nm} of 12.5). Culture samples corresponding to ca. Two Hundred and Forty Milligram of biomass wet weight were directly quenched in liquid nitrogen. The resulting frozen pellet was gently thawed on ice and spun down at 4700 × g for 5min at 0°C. Pellets were then resuspended in 1.2mL of ice-cold AE buffer (50mM sodium acetate and 10mM EDTA, pH 5.0), followed by addition of 1.2mL of acid phenol/chloroform/isoamyl alcohol mix and 0.12mL 10% sodium dodecyl sulfate. The resulting mix was vortexed for 30s and incubated for 5min at 65°C. After homogenizing for 30s by vortexing, 800 µL aliquots were distributed in RNase-free screw-cap tubes [221]. After centrifugation (15min at 10,000 × g), the aqueous phase was transferred to a new tube containing 0.4mL of acid phenol/chloroform. The mix was vortexed for 30s, centrifuged (15min at 10,000 × g) and the aqueous phase was again transferred to a new tube. RNA was then ethanol precipitated and re-suspended in RNAse-free water. Prior to cDNA synthesis, purity, concentration, and integrity of the RNA in the samples was assessed with the Nanodrop (Thermo Fisher Scientific), Qubit (Thermo Fisher Scientific), and Tapestation 220 with RNA Screen Tape (Agilent Technologies), respectively, according the manufacturers' recommendations. cDNA libraries were prepared using the TruSeq RNA V2 kit (Illumina) and sequenced on HISeq 2500 (Illumina) at Novogene (HK) Company Ltd (Hong Kong, China).

Transcriptome analysis

Libraries with 300 bp insert size were paired end sequenced (150 bp). Duplicate biological samples were processed, generating an average sequence quantity of 23.7M reads per sample. Reads were aligned to the Oxford Nanopore CBS 12357^T reference assembly using a two-pass STAR [222] procedure. In the first pass, splice junctions were assembled and used to inform the second round of alignments. Introns between 15 and 4,000 bp were allowed, and soft clipping was disabled to prevent low-quality reads from being spuriously aligned. Ambiguously mapped reads were removed from the dataset. Expression level for each transcript were quantified using htseq-count [223] in union mode. Fragments per kilo-base of feature (gene) per million reads mapped (FPKM) values were calculated by "Applying the rpkm method" from the edgeR package [224, 225]. Differential expression analysis was performed using DESeq [226]. Transcript data can be retrieved at the Genome Omnibus Database (GEO: https://www.ncbi.nlm.nih.gov/geo/) under accession number: GSE117246.

Analytical methods

Optical densities of yeast cultures were measured with a LibraS11 spectrophotometer (Biochrom, Cambridge, UK) at a wavelength of 660nm. Biomass dry weight was measured by filtering 10mL culture samples over pre-weighed nitrocellulose filters with a pore size of 0.45µm. Filters were washed with 10mL water, dried in a microwave oven (20min at 350W) and reweighed. Each measurement was performed in duplicate. For glucose, maltose, maltotriose, and ethanol analysis, culture samples were centrifuged 5min at 10,000g and supernatants were analyzed by high-performance liquid chromatography (HPLC) analysis on an Agilent 1260 HPLC equipped with a Bio-Rad HPX 87H column (Bio-Rad, Hercules, CA). Elution was performed at 65°C with 5mM H_2SO_4 at a flow rate of 0.8mL min^{-1}. Detection was by means of an Agilent refractive-index detector and an Agilent 1260 VWD detector.

Viability measurements using fluorescence-assisted cell sorting

Cultures were analyzed on a BD FACSAria™ II SORP Cell Sorter (BD Biosciences, Franklin Lakes, NJ) equipped with 355, 445, 488, 561, and 640nm lasers and a 70µm nozzle, and operated with filtered FACSFlow™ (BD Biosciences). Correct cytometer performance was evaluated prior to each experiment by running a CST cycle with corresponding CS&T Beads (BD Biosciences). Drop delay for sorting was determined by running an Auto Drop Delay cycle with Accudrop Beads (BD Biosciences). Morphology of the cells was analyzedby plotting forward scatter (FSC) against side scatter (SSC). Ninety-Six single cells were sorted onto 96-well format Nunc omnitray (Thermo Scientific) plates containing SMG agar using a “single cell” sorting mask, corresponding to a yield mask of 0, a purity mask of 32 and a phase mask of 16. Viability was measured as the average percentage of sorted cells able to form a colony after 48h incubation at 30°C on three triplicate plates.

Results

A high-quality *S. eubayanus* genome assembly with 330kb of previously unexplored sequence including four *MAL* loci

Owing to advances in genome sequencing technology, the quality of genome sequence data of *S. eubayanus* CBS 12357^T/ FM1318 has gradually improved [34, 74, 75, 78]. The currently available reference sequence is based on second generation sequencing technology (Illumina generated data), obtained from libraries with different insert sizes that were co-assembled into a 11.66Mb genome, comprising 144 contigs forming 22 scaffolds. While representing an important resource for research on *S.*

eubayanus and *S. pastorianus*, this most advanced draft genome sequence is incomplete [75]. In particular multiple repeated regions, such as subtelomeric regions, are not yet fully resolved due to limitations of short-read sequencing technology. In total, approximately 122kb of the scaffolded genome remain undefined.

To generate a near-complete, chromosome-level de novo assembly of *S. eubayanus* CBS 12357^T, we used long-read sequencing with third-generation single-molecule technology (Oxford Nanopore Technology MinION platform). A single flow cell was used to generate 3.3 Gb of sequence reads, with an average read length of 8kb and an estimated average error rate of 9.6%. These data represented a genome coverage of 135-fold of the estimated diploid genome size (24Mb). An assembly exclusively based on the MinION reads was generated with the Canu program [214]. This assembly yielded 19 contigs, which is 8-fold fewer than obtained in the short-read-only assembly of the latest CBS 12357^T draft genome [75]. In the MinION-based assembly, the mitochondrial genome and all chromosomes except for CHRXII were assembled as single contigs. CHRXII was manually reconstructed by joining three contigs, with a 1,000N residues gaps introduced between the contigs. The sequence discontinuity was caused by the inability of the assembly software to handle the highly repetitive DNA organization of the rDNA locus (Figure 3). This approach yielded a nearly complete 11.9Mb genome assembly.

Prior to annotation, the assembly based on MinION sequencing data was "polished" with additional Illumina sequencing data using Pilon [215]. *In fine* this polished genome assembly included 330kb of sequence which were not assembled in the previous genome assembly. With the exception of a region on chromosome XII that corresponded to a partially reconstructed rDNA locus, additional sequences were mainly located in the subtelomeric regions (Figure 3A). A total of 5,444 ORFs were annotated, including 41 previously unassembled ORFs [75]. Additionally, 60 ORFs were modified relative to the earlier draft genome, often leading to a redefinition of start and stop codons (Figure 3A, Table S6). For 65 of the 101 new genes, a paralog or an identical copy of the gene had already been assembled at a different location in the previous assembly. Gene ontology analysis using Fischer's Exact Test revealed an overrepresentation among the new genes of three GO categories related to sugar transport and cell wall (Table 3). This is not that surprising since, subtelomeres are acknowledged to be the major chromosomal regions involved functional evolution as they are sites for large rearrangement in term of structure, gene content and copy number (not only gain but also lossof-function variants; [97, 227].

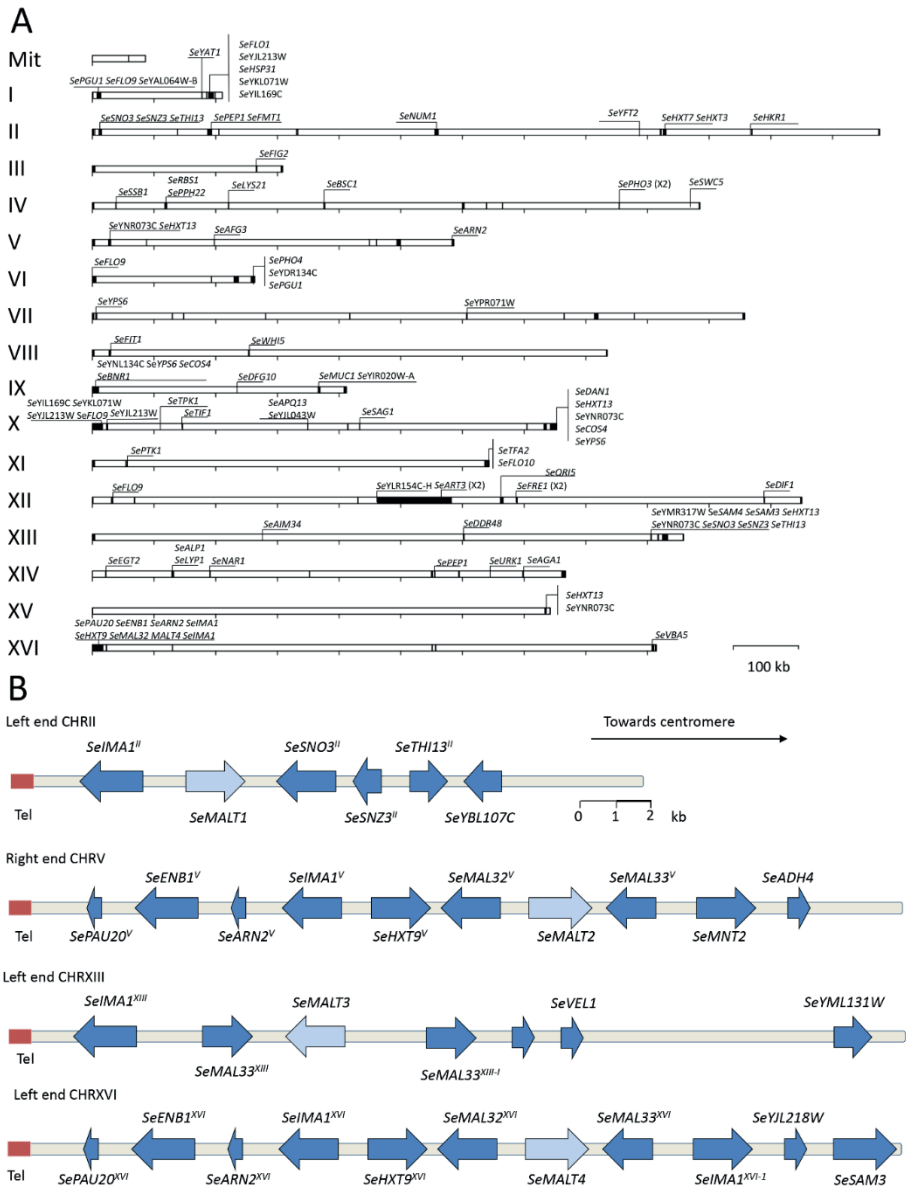

Figure 3: High-quality *S. eubayanus* genome assembly with 330 kb of unexplored sequence including four MAL loci. **A** Representation of the assembled *S. eubayanus* chromosomes, the black boxes denote newly added sequences. New annotated open reading frames and gene entries modified relative to the earlier draft genome [75] often leading to a redefinition of start and stop codons. **B** Organization of subtelomeric regions including maltose metabolism genes. Arrows denote the direction of transcription. The label "Tel" indicates the position of the telomere. The *SeMALT* genes are indicated in light blue. The gene and interval sizes are approximately to scale

Table 3: Overrepresented GO functional categories among 101 newly identified genes in a MinION - sequencing based *S. eubayanus* CBS 12357 genome assembly annotation.

GO	Function	Genes	P-value*
GO:0031225	anchored component of membrane (15)	*SeFLO1*, *SeFLO9* (X4), Se*FLO10*, *SeFIG2*, *SeYPS6* (X3), Se*SAG1*, *SeDAN1*, *SeEGT2*, *SeAGA1*,	1.78E-10
GO:0005618	cell wall (15)	*Se*YIL169C	7.04E-09
GO:0008645	hexose transport (8)	*SeHXT7*, *SeHXT3*, *SeHXT13* (X4), Se*MAL31/SeMALT4*, *SeHXT9*	4.77E-4

*Enrichment of functional categories was assessed by Fisher's exact test using Bonferroni correction.

Four subtelomeric regions harbored complete sequences of putative maltose transporters. Two of these, which contained *SeMALT1* and *SeMALT3*, showed structural features that differed from those of canonical *S. cerevisiae MAL* loci. The CHRII locus only contained a transporter gene (*SeMALT1*) while the CHRXIII "*SeMAL* locus" consisted of a transporter gene (*SeMALT3*) flanked by two non-identical genes that strongly resembled the *S. cerevisiae* regulator genes *MAL33* and *MAL63* (Figure 3B). In contrast, the *S. eubayanus MAL* loci on CHRV and CHRXVI showed the same organization as the well described *S. cerevisiae MAL* loci. Starting from their telomeric ends, they contained a maltase gene (*SeMAL32*), followed by the transporter gene (*SeMALT2* on CHRV and *SeMALT4* on CHRXVI), which shared a bi-directional promoter with the maltase gene, and a *MAL* regulator gene (Figure 3B, Figure S8). Similarity between the right-arm CHRV and left-arm CHRXVI subtelomeric regions extended beyond the *SeMAL* genes, with a sequence identity of 94% and shared gene synteny over a 20kb region (Figure 3B). The fully assembled *SeMALT4* gene shared 99.7% identity with *SeMALT2*, from which it differed by only five nucleotides. None of these five nucleotide variations affected the predicted amino acid sequence of the encoded transporters.

Systematic deletion of *MALT* genes revealed that *SeMALT2/SeMALT4* are essential for growth on maltose

To explore the contribution of the four *S. eubayanus MALT* genes to maltose consumption, deletion strains were constructed. Because the high sequence similarity of *SeMALT2* and *SeMALT4* complicated individual deletion of these genes, three strains were constructed with either a single deletion of *SeMALT1* or *SeMALT3* or a double deletion of *SeMALT2* and *SeMALT4* (Figure 1). The option offered by CRISPR-Cas9 to simultaneously delete of multiple gene copies in a single transformation step [206] is especially helpful in diploid strains such as *S. eubayanus* CBS 12357^T. To explore the use of this methodology in *S. eubayanus*, we used a broad-host-range yeast plasmid for co-expression of Spcas9 and a cassette encoding a ribozyme-flanked gRNA, which was successfully used in the Saccharomycotina yeasts *S. pastorianus* [171], *Kluyveromyces* sp. and *Ogataea* sp. [204].

Cloning of specific gRNA cassettes targeting *SeMALT1*, *SeMALT2/T4*, and *SeMALT3* in pUDP002 resulted in pUDP062, pUDP063, and pUDP064, respectively. These plasmids were then transformed into *S. eubayanus* CBS 12357^T, either alone or in combination with a 120-bp double stranded repair DNA fragment for the targeted *SeMALT* gene (Figure 1). In the absence of a repair fragment, transformation with a gRNA-expressing construct was expected to be fatal if both gene copies were cut, unless both breaks were repaired by non-homologous end joining (NHEJ) of the induced double strand breaks. However, transformation of *S. eubayanus* CBS 12357^T with pUDP062, pUDP063 or pUDP064 alone yielded 1100, 128, and 9 transformants, respectively. These numbers were not substantially different from those observed upon co-transformation of the corresponding repair fragments (3000, 114, and 13 colonies, respectively). Based on a set of 30 transformants, genome editing with the gRNA$_{SeMALT1}$ yielded the lowest frequency of transformants in which both gene copies were deleted (3%). The *SeMALT3* gRNA performed better with a 7% frequency out of 13 transformants tested, while the gRNA targeting *SeMALT2/T4* showed an efficiency of 40% of accurate deletion of both copies of the two genes out of a set of eight transformants.

Table 4: Specific growth rates (h^{-1}) of *S. eubayanus* CBS 12357^T [74], *S. eubayanus* (*Se*) maltose transporter deletion mutants, *S. cerevisiae* (*Sc*) strains overexpressing individual *S. eubayanus* maltose transporter genes and the maltose-consumption-deficient host strain *S. cerevisiae* IMZ616 [198]. *S. eubayanus* strains were grown on SMG and SMM media at 20 ^oC. *S. cerevisiae* strains were grown on SM_UG and SM_UM at 20 ^oC. Data represent average and standard deviation of three biological replicates. "*malΔ*" denotes the following genotype *mal1Δ mal2Δ mal3Δ mph2Δ mph3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ.*

Strain	Species	Relevant genotype or phenotype	Specific growth rate (h^{-1})	
			Glucose	Maltose
CBS 12357^T	*Se*	*SeMALT1 SeMALT2 SeMALT3 SeMALT4*	0.24 ± 0.003	0.17 ± 0.001
IMK816	*Se*	*SemalT1Δ* Se*MALT2 SeMALT3 SeMALT4*	0.22 ± 0.001	0.17 ± 0.000
IMK817	*Se*	*SeMALT1* Sem*alT2Δ* Se*MALT3 SemalT4Δ*	0.22 ± 0.002	0.002 ± 0.000
IMK818	*Se*	*SeMALT1 SeMALT2 SemalT3Δ SeMALT4*	0.22 ± 0.001	0.17 ± 0.002
IMZ616	Sc	*"malΔ"*	0.19 ± 0.002	0.00 ± 0.000
IMX1253	*Sc*	*"malΔ" ScTEF1$_{pr}$-SeMALT1-ScCYC1$_{ter}$ ScMAL12*	0.20 ± 0.003	0.13 ± 0.002
IMX1254	*Sc*	*"malΔ" ScTEF1$_{pr}$-SeMALT2-ScCYC1$_{ter}$ ScMAL12*	0.18 ±0.002	0.13 ± 0.001
IMX1255	*Sc*	*"malΔ" ScTEF1$_{pr}$-SeMALT3-ScCYC1$_{ter}$ ScMAL12*	0.19 ±0.002	0.13 ± 0.004
IMX1365	*Sc*	*"malΔ" ScTEF1$_{pr}$-ScMAL11-ScCYC1$_{ter}$ ScMAL12*	0.19 ±0.001	0.099 ± 0.005

The resulting *S. eubayanus* deletion strains IMK816 (*SemalT1Δ*), IMK817 (*SemalT2Δ SemalT4Δ*), and IMK818 (*SemalT3Δ*), as well as the wild-type strain CBS 12357^T, were grown in SMG and SMM media. While specific growth rates of all four strains in SMG were the same (0.22 h^{-1} at 20°C, Table 4), strain IMK187 (*SemalT2Δ SemalT4Δ*)

did not grow on maltose (Figure 4). Conversely, strains IMK816 and IMK818 exhibited the same specific growth rate on maltose as the reference strain (0.17 h^{-1} at 20°C, Table 4). These data suggested that only *SeMALT2* and/or *SeMALT4* only contributed to growth on maltose of wild- type *S. eubayanus* CBS 12357^{T}.

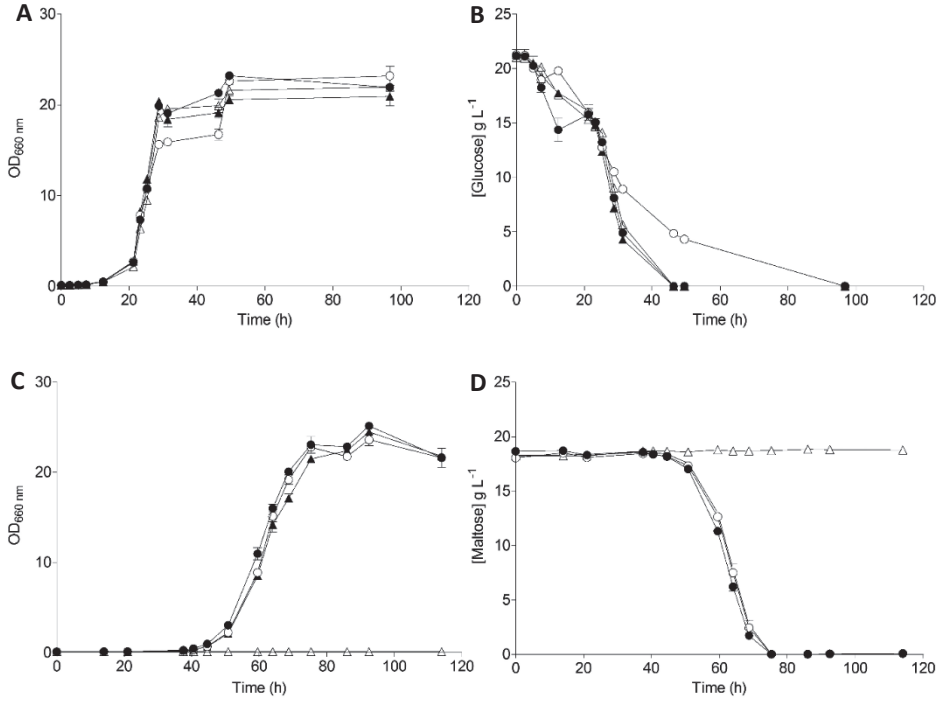

Figure 4: **Characterization of the *S. eubayanus* strains (●) CBS 12357^{T}, (▲) IMK816 (*SemalT1*Δ), (△) IMK817 (*SemalT2Δ SemalT4Δ*) and (○) IMK818 (*SemalT3*Δ) during growth on glucose and maltose as sole carbon source**. *S. eubayanus* strains were grown on SMG or SMM at 20 °C. Growth on glucose **A** and on maltose **C** was monitored based on optical density measurement at 660 nm (OD_{660nm}). Concentrations of glucose **B** and maltose **D** in culture supernatants were measured by HPLC. Data are presented as average and standard deviation of three biological replicates.

SeMALT1, *SeMALT2/T4*, and *SeMALT3* all support maltose uptake

For further functional analysis, *SeMALT1*, *SeMALT2*, and *SeMALT3* were expressed in a maltose-transporter negative *S. cerevisiae* strain background. *SeMALT4* was not included in this comparison, as it encodes a protein with the same amino acid sequence as *SeMALT2*. The maltose-negative *S. cerevisiae* strain IMZ616 originates from *S. cerevisiae* CEN.PK102-3A, which carries three *MAL* loci (*MAL1*, *MAL2*, and *MAL3*) [197]. To eliminate growth on α-glucosides, these three *MAL* loci, *MPH2*, and *MPH3* as well as the αglucoside hydrolase-encoding genes *SUC2* and *IMA1-5* were deleted, yielding *S. cerevisiae* IMK291 [198]. Introduction of *cas9* into this strain yielded IMZ616 [200].

Restoration of growth on maltose of *S. cerevisiae* IMZ616 requires simultaneous expression of a maltose transporter and a maltase. Therefore, the three *S. eubayanus* transporter genes were cloned behind the constitutive *ScTEF1* promoter and, together with an expression cassette for the *ScMAL12* maltase gene, integrated at the *SGA1* locus of *S. cerevisiae* IMZ616 (Figure 2). The resulting *S. cerevisiae* strains IMX1253, IMX1254, IMX1255, which expressed *SeMALT1*, *SeMALT2*, and *SeMALT3*, respectively, were grown on SM_UM. As expected, the host strain IMZ616 did not show any growth on maltose, while the three *SeMALT*-expressing strains showed different growth profiles on this disaccharide. Strain IMX1255 (*SeMALT3*) resumed growth after a lag phase of ~10h and consumed half of the maltose supplied (Figure 5). Strains IMX1253 (*SeMALT1*) and IMX1254 (*SeMALT2*) showed lag phases of 100 and 250h, respectively. However, after these lag phases, maltose was consumed. Strain IMX1254 (*SeMALT2*) consumed 75% of the supplied maltose in 150h. In the same conditions the control strain IMX136 5 co expressing *ScAGT1* and *ScMAL12* showed a short lag phase of 10h and that was immediately followed by exponential growth, IMX1365 reached stationary phase and full maltose consumption in less than 100h a performance comparable to the IMX1255 (*SeMALT3*) (Figure 5).

These delayed growth phenotypes of the *SeMALT*-expressing strains resemble those observed during growth on lactose of *S. cerevisiae* strains expressing the β-galactosidase (*LAC4*) and the lactose permease (*LAC12*) genes of *Kluyveromyces marxianus* and *Kluyveromyces lactis* [228, 229]. In those studies, lactose utilization first had to be improved by laboratory evolution which resulted in lowering copy number of the plasmid harboring the permease and hydrolase genes as well as a short internal deletion located in the bi-directional promoter driving expression of the two genes [229]. The same way, an adaptation step have been included in the workflow for complementation of the hexose transporter null (*hxt^0*) strain EBYWV4000 with human glucose transporter (*GLUT*) [230] suggesting that swapping transporter or implementing new assimilatory pathway remains nontrivial.

When, at the end of a first round of batch cultivation on maltose, cells of the *SeMALT*-expressing strains were transferred to fresh maltose medium, they all showed instantaneous growth at a specific growth rate of 0.13 h^{-1} (Table 4). Under the same

conditions, *S. eubayanus* CBS 12357^{T} grew on maltose at a specific growth rate of 0.17 h^{-1} (Figure 5, Table 4). Even after transfer to fresh maltose medium, none of the heterologously expressed *SeMALT* transporters enabled full maltose consumption in these cultures. Similarly to the first cycle of batch cultivation on maltose, strain IMX1254 (*MALT2*) consumed 75% of the maltose supplied, while strains IMX1255 (*SeMALT3*) and IMX1253 (*SeMALT1*) consumed ca. 50 and 35 percentage even after prolonged incubation, none of the *S. cerevisiae* strains expressing an *SeMALT* gene nor *S. eubayanus* CBS 12357^{T} showed growth on maltotriose, while the positive control IMX1365 (*ScAGT1*) showed growth at a rate of 0.08 ± 0.007 h^{-1} (Figure S9).

In contrast to the deletion study, which suggested that only SeMalt2 and/or SeMalt4 were able to transport maltose, heterologous expression in the Mal^{-} *S. cerevisiae* strain IMZ616 showed that all four transporter genes encode transporters that, in combination with a maltase, allow growth on maltose.

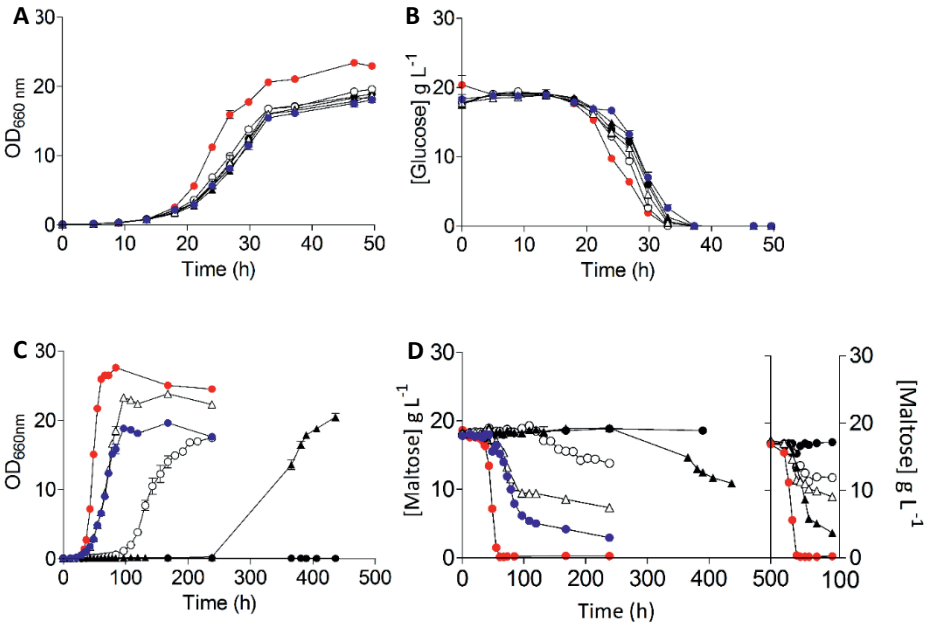

Figure 5: **Characterization of the *S. cerevisiae* strains (●) IMZ616 [200], (○) IMX1253 (Sc*TEF1*$_{pr}$-*SeMALT1*-*ScCYC1*$_{ter}$), (▲) IMX1254 (Sc*TEF1*$_{pr}$-*SeMALT2*-*ScCYC1*$_{ter}$), (△) IMX1255 (Sc*TEF1*$_{pr}$-*SeMALT3*-*ScCYC1*$_{ter}$), (●) IMX1365 (*ScTEF1*$_{pr}$-*ScMAL11*-*SeCYC1*$_{ter}$) and (●) *S. eubayanus* CBS 12357^{T}.** The strains were grown in SM$_{U}$G and SM$_{U}$M at 20 °C. Growth on glucose **A** and on maltose **C** was monitored based on optical density measurement at 660 nm (OD$_{660nm}$). Concentrations of glucose **B** and maltose **D** in culture supernatants were measured by HPLC. Panel **D** shows the data of two consecutive batches. Data are presented as average and standard deviation of three biological replicates.

Slow growth of *SeMALT*-expressing *S. cerevisiae* strains is not caused by maltose accelerated death

Since maltose is imported by proton symporters energized by the plasma-membrane proton-motive force [231, 232], an unrestricted influx of maltose can lead to a fast influx of protons [233]. Unless the resulting rate of proton influx can be countered by the proton-pumping plasma-membrane ATPase (Pma1; [231]), dissipation of the proton motive force and cytosolic acidification can cause maltose-induced cell death. Indeed, pronounced maltose-accelerated death has been observed *S. cerevisiae* evolved an increased maltose transport capacity [233]. To test whether this phenomenon was responsible for the observed delayed growth of *S. cerevisiae* strains expressing *S. eubayanus* maltose transporters, the corresponding *S. cerevisiae* strains (IMX1243, IMX1254, and IMX1255) were first grown on SMG. Upon reaching late exponential phase, cells after washing were transferred to SM medium (without C-source) to give an OD_{660} of 1.0. The resulting cell suspension was then sampled before and 30, 120, and 270min after addition of 20g.L^{-1}maltose. From each sample, 96 cells were sorted using gated Forward scatter signal and side scatter signal intensities on SMG medium and the viability was estimated based on the number of growing colonies. Neither the three *SeMALT*-expressing *S. cerevisiae* strains, nor *S. eubayanus* CBS 12357^T or the Mal$^-$ *S. cerevisiae* IMZ616 showed a decreased viability over a period of 270min exposure to maltose (Figure 6). This result indicated that delayed growth of the *SeMALT*-expressing *S. cerevisiae* strains was not due to maltose accelerated death.

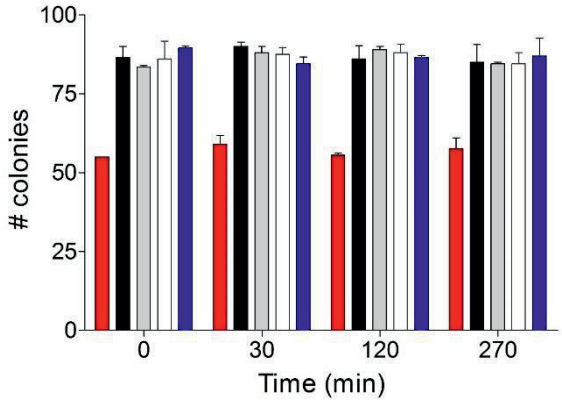

Figure 6: Cell viability after exposure to maltose of glucose-pregrown cultures of *S. eubayanus* CBS 12357^T (■), *S. cerevisiae* IMZ616 (■), IMX1253 (*SeMALT1*) (■), IMX1254 (*SeMALT2*) (□) and IMX1255 (*SeMALT3*) (■). Cells from glucose-grown batch cultures were resuspended in SM. Prior addition of 20 g L^{-1} of maltose, the initial viability was measured by sorting 96 cells per strain on SMG plates. The SMM cultures were sampled after 30, 120 and 270 minutes. The viability data are represented as averages ± mean deviations of three independent experiments for each strain.

In contrast to *SeMALT2/T4*, the transporter genes *SeMALT3* and *SeMALT1* were not efficiently transcribed in maltose grown cells

The inability of the double deletion mutant *S. eubayanus* IMK817 (*SemalT2Δ - SemalT4Δ*) to grow on maltose, despite the demonstration that *SeMALT1* and *SeMALT3* have the potential to encode functional maltose transporters, might indicate that *SeMALT1* and *SeMALT3* are not expressed in maltose-grown cultures. To investigate the impact of carbon sources on genome-wide transcript profiles and, more specifically, on transcriptional regulation of maltose metabolism genes, duplicate cultures of the *S. eubayanus* wild-type strain CBS 12357^T were grown on SMG and SMM and sampled in mid-exponential phase (OD_{660nm} = 12.5 ± 1.0). After mRNA isolation and processing, cDNA libraries were sequenced with Illumina sequencing technology. cDNA sequencing reads were mapped onto the newly annotated *S. eubayanus* CBS 12357^T genome assembly and used to calculate FPKM (fragments per kilobase of feature (gene) per million reads mapped) expression values. FPKM results represent normalized expression values that take into account gene length and sequencing depth. Statistical analysis showed that 125 genes were differentially expressed in the glucose- and maltose-grown cultures with a fold difference > 4 (Table S7). All four *S. eubayanus MALT* transporters were significantly upregulated during growth on maltose and three (*SeMALT2*, *SeMALT4*, and *SeMALT1*) were found among the ten most upregulated genes (Table 5). *SeMALT2* and *SeMALT4* exhibited 262- and 244-fold higher transcript levels during growth on maltose than during growth on glucose. *SeMALT1* and *SeMALT3* represented a substantially lower fold-difference between maltose- and glucose-grown cultures (67- and 6.8- fold, respectively; Figure 7, Table 5). The most pronounced difference between *SeMALT2/SeMALT4* and *SeMALT1/SeMALT3* concerned their expression level. The FPKM value of *SeMALT1* in maltose grown cultures was 48-fold lower than that of *SeMALT2* and *SeMALT4* value ($FPKM_{SeMALT1}$ = 30; $FPKM_{SeMALT2}$ = 1,683, $FPKM_{SeMALT4}$ = 1451). Similarly, *SeMALT3* exhibited a FPKM value of only 200. In the same analysis, *SeACT1* and the glycolytic gene *SeTDH3*, genes commonly used as internal standard in transcript analysis exhibited substrate-independent FPKM values of 1600 and 6000, respectively. The maltase genes that were physically associated to *SeMALT2* and *SeMALT4* (Figure 3) were also strongly overexpressed in maltose-grown cultures, representing the highest upregulation fold difference in expression in glucose- and maltose-grown cultures of *S. eubayanus* (Table 5). This result confirms the functionality of the bidirectional promoters controlling the maltase and transporter genes in the *S. eubayanus MAL* loci harboring *SeMALT2* and *SeMALT4*.

Table 5: Transcript level of the 15 most strongly upregulated genes in maltose-grown *S. eubayanus* CBS 12357^T. FPKM gene expression values of *S. eubayanus* CBS 12357^T grown on SMG (glucose) and SMM (Maltose) were calculated from duplicate RNA seq experiments (2 x 150bp; 7.1 Gb) using the "Applying rpkm" method from the EdgeR package [224]. *p*-values were calculated using DESeq and adjusted for multi-testing. The descriptions were based on similarity with *S. cerevisiae* orthologs.

CHR	Coordinates	Gene name	Description	FPKM		Fold-change	adjusted p-value
				Glucose	Maltose		
CHRXVI	13815-15572	*SeMAL32^{XVI}*	Maltase (alpha-D-glucosidase)	9.9 ± 0.2	5218.4 ± 365	525.6	0
CHRV	569448-571205	*SeMAL32^{V}*	Maltase (alpha-D-glucosidase)	9.6 ± 0.0	4620.4 ±3 84	481.3	0
CHRV	566765-568606	*SeMALT2*	Maltose permease-member of the 12 TMB domain family of sugar transporters	5.5 ± 0.4	1451 ± 39	262.7	0
CHRXVI	16413-18254	*SeMALT4*	Maltose permease-member of the 12 TMB domain family of sugar transporters	6.9 ± 0.4	1683.8 ± 30	244.5	0
CHRIV	971754-973529	*SeDAK2*	Dihydroxyacetone kinase required for detoxification of dihydroxyacetone	19.2 ± 0.6	3342.3 ± 171	174.1	0
CHRII	8497-10338	*SeMALT1*	Maltose permease-member of the 12 TMB domain family of sugar transporters	0.5 ± 0.0	30.8 ± 1	67.7	3.2E-82
CHRXVI	25036-25626	*SeYJL218W*	Putative acetyltransferase	8.3 ± 2.3	528.3 ± 4	63.9	2.1E-246
CHRIV	968740-970695	*SeYFL054C*	Putative channel-like protein; similar to Fps1p	10.7 ±2.2	529.6 ± 22	49.3	0
CHRII	5766-7535	*SeIMA1^{II}*	Isomaltase, α -1,6-glucosidase; required for isomaltose utilization	1.6 ± 0.1	66.6 ± 5	41.7	2.7E-150
CHRIV	974654-975250	*SeREE1*	Cytoplasmic protein involved in the regulation of enolase	27.0 ± 1.1	1079.8 ± 76	40.0	2.3E-293
CHRV	564762-565970	*SeMAL33^{V}*	MAL-activator protein	10.0 ± 1.2	196.3 ± 17	19.6	4.4E-171
CHRIV	512918-513952	*SeYRO2*	Protein with a putative role in response to acid stress	128.1 ±6.9	2348.5 ± 110	18.3	2.5E-238
CHRXVI	732395-734113	*SeHXT13^{XVI}*	Putative transmembrane polyol transporter	14.2 ± 3.9	202.2 ± 19	14.3	3.4E-113
CHRIV	552424-553068	*SeHSP26*	Small heat shock protein (sHSP) with chaperone activity	137.5 ± 19.5	1520.3 ±33	11.1	1.2E-147
CHRVII	934199-934486	*SeSPG1*	Protein required for high temperature survival during stationary phase	6.3 ± 1.6	65.3 ± 5	10.3	2.3E-31

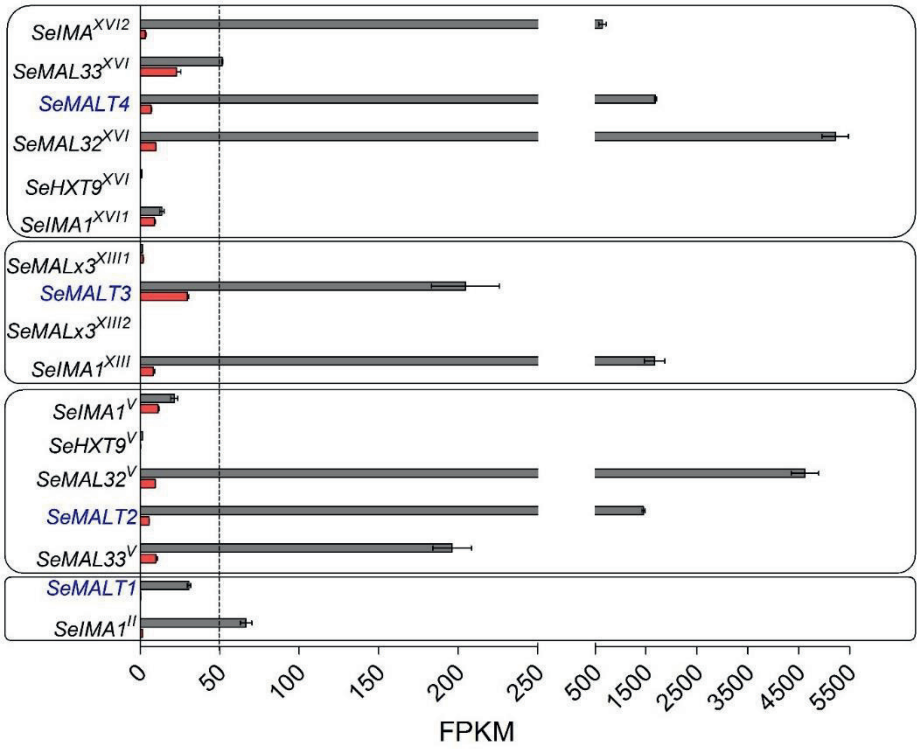

Figure 7: Expression of the maltose metabolism associated genes in *S. eubayanus* CBS 12357^{T}. Transcript levels of maltose metabolism genes located at subtelomeric regions of CHRII, V, XII and XVI. FPKM gene expression values of *S. eubayanus* CBS 12357^{T} grown on SMG (glucose, (■)) and SMM (maltose, (■)) at 20 ^{o}C were calculated from duplicate RNA seq experiments (2 x 150bp; 7.1 Gb) using the "Applying rpkm" method in the EdgeR package [224]. Data represent average and mean deviation of two biological replicates.

Discussion

S. eubayanus is not only a key contributor to the hybrid genomes of current *S. pastorianus* lager brewing strain and a basis for developing new brewing yeast hybrids [167] but is also directly used for brewing specialty lager beer [234]. This study provided an upgraded, near-complete genome sequence of the *S. eubayanus* type strain CBS 12357^{T}and the first characterization of its four maltose transporter genes.

Complete and accurate de novo assembly of eukaryotic genomes has long been a major challenge. While short-read, high-coverage technologies such as the popular Illumina platforms enable accurate sequencing and assembly of unique genomic sequences, they cannot resolve repetitive sequences. As previously demonstrated for *S. cerevisiae* CEN.PK113-7D [93, 101, 235], use of the Oxford Nanopore MinION platform

generated long-reads that enabled a near-complete assembly of 15 of the 16 chromosomes of *S. eubayanus* CBS 12357^T (Figure 3A). Correct assembly of subtelomeric regions, which are known for their high sequence redundancy, was especially important in the context of this study in view of the subtelomeric location of the four *MAL* loci in this strain (Figure 3B). Despite the massive sequencing coverage yielded by a single flow cell, which was invaluable for genome scaffold construction, the intrinsic higher error rate of MinION sequencing [236] required polishing with additional Illumina sequencing data. Although highly effective, as illustrated by the complete assembly of the four *MAL* loci and the use of resulting high-quality genome sequence information for transcriptome analysis, this approach could not correct all errors. In particular, INDELs causing omissions of single nucleotides in homopolymer regions were left in the final assembly, which is a known pitfall of the single molecule nanopore sequencing (Oxford Nanopore Technology MinIOn). Manual curation and, in particular, validation of relevant sequences by Sanger sequencing will be required to further refine the current genome sequence of *S. eubayanus* CBS 12357^T.

Also, the first application of CRISPR-Cas9-assisted genome editing in *S. eubayanus* reported in this study was greatly facilitated by the availability of an accurate genome sequence. Resolution of the sequences of the four *SeMALT* genes enabled optimization of the gRNA spacer selection, thereby minimizing the risk of undesirable off-target events. Although targeting efficiencies of the employed ribozyme-flanked-gRNA expression system [171], which ranged from 3 to 40% were not ideal, Cas9-assisted gene deletion offered clear advantages over traditional methods that rely on a double crossover event that inserts a DNA fragment containing a selection marker in the recipient strain's genome [237, 238]. Cas9-assisted genome editing in *S. eubayanus* did not make use of a marker cassette and, most importantly, enabled simultaneous marker-free editing both alleles of the *SeMALT1* or the *SeMALT3* gene. In the case of *SeMALT2* and *SeMALT4*, a single transformation was even sufficient to delete both alleles of two genes [170, 206]. Achieving these objectives with conventional techniques would have been extremely time consuming as multiple rounds of transformation and marker recovery would be required. Depending on consumer acceptance and regulations in place [179, 239], marker-free, Cas9-assisted genome editing of *S. eubayanus* may be combined with the generation of new Saccharomyces hybrids to accelerate development to novel brewing strains.

Cas9-mediated gene disruption and transcriptome analysis in *S. eubayanus* CBS 12357^T showed that, in maltose-grown cultures, *SeMALT2* and *SeMALT4* were predominantly responsible for maltose uptake. These transporters are located within two nearly identical (97% identity) *SeMAL* loci that strongly resemble canonical *S. cerevisiae MAL* loci [130, 138, 191]. Although two other genes, *SeMALT3* and *SeMALT1*, could restore growth upon their expression in the Mal$^-$ *S. cerevisiae* strain IMZ616, their low expression levels in *S. eubayanus* CBS 12357^T were apparently not sufficient to support growth on maltose when *SeMALT2* and *SeMALT4* were both deleted (Figures 3, 7). This study did not

provide new insights into the origin of *MTT1*/*MTY1* [131, 132] and *SeAGT1* [34, 83, 145] in industrial *S. pastorianus* strains as, consistent with earlier observations [75], no genes with strong sequence similarity to these maltose transporter genes were identified in the improved genome sequence of *S. eubayanus* CBS 12357^T.

The low expression levels of *SeMALT1* and *SeMALT3* may be related to their genomic context, as they were located in atypical *SeMAL* loci on CHRII and CHRXIII, respectively (Figure 3B). Assuming that these two atypical *SeMAL* loci evolved from a complete *MAL* locus, loss of the maltase gene may have disrupted the bi-directional promoter that, in *S. cerevisiae*, controls expression of both the maltose and the maltose transporter genes [240]. In *S. cerevisiae*, the maltose regulator Mal63 binds two regulatory sites (5′MGSN$_9$MGS3′) located between positions −465 and −579 in the region separating the two divergent genes [152]. While two of these elements were also found in the promoter regions of *SeMALT2* and *SeMALT4* (Figure S8), the *SeMALT1* and *SeMALT3* promoters each harbored only a single element (Figure S8). Alternatively, low expression of *SeMALT1* and *SeMALT3* in maltose-grown cultures may reflect a sub-functionalization that, during evolution, led to a different regulation and/or catalytic properties of the encoded transporters [241, 242]. Indeed, such a subfunctionalization has been experimentally reconstructed for yeast α-glucoside hydrolases [243].

Apart from maltose metabolism related genes, transcriptome analysis reveled the overexpression of further genes that are suggested to be involved in stress tolerance. The stress that leads to this up regulation is caused by the switch of carbon sources in the growth media (from glucose in precultures to maltose as sole carbon source) as well as the fermentation of maltose at 20°C accompanied by the production of ethanol and the acidification of the growth medium.

None of the four *SeMALT* genes identified in *S. eubayanus* CBS 12357^T were found to encode a functional maltotriose transporter. Although, the *S. eubayanus* Patagonian lineage is unlikely to have contributed the *S. eubayanus* subgenome of *S. pastorianus* lager brewing strains [83], this observation would be consistent with the notion that *S. cerevisiae* has contributed the vital ability to ferment this trisaccharide, which is abundantly present in wort as in contrast multiple *S. cerevisiae* ale strains mainly issued from the beer 1 and 2 groups have been shown to use this sugar [70]. However, CBS 12357^T is a representative of sole the Patagonia B group, one of five groups defined based of the phylogenetic distribution of *S. eubayanus* strains isolated so far [110]. Therefore, we cannot at all exclude the possibility that this brewing relevant phenotypic trait of lager yeast would originate from the *S. eubayanus* parent. The only elements so far that could tilt toward this hypothesis are very fragmented sequencing data of an isolate from the Holartic group, that suggested occurrence of *S. eubayanus* ortholog of the *S. cerevisiae AGT1/MAL11* gene [34]. Therefore, the resource and methodology used in this study paved the way for further exploration of the diversity of *S. eubayanus* population and elucidation of *S. eubayanus* parental lineage of *S. pastorianus*.

Acknowledgements

This project was funded by the Seventh Framework Programme of the European Union in the frame of the SP3 people support for training and career development of researchers (Marie Curie), Networks for Initial Training (PITN-GA-2013 ITN-2013-606795) YeastCell (https://yeastcell.eu/) and the BEBasic R&D Program (http://www.be-basic.org/), which was granted an FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I). We thank Alex Salazar, Niels Kuijpers (Heineken Supply Chain B.V.) and Jan-Maarten Geertman (Heineken Supply Chain B.V.) for their support and Arthur Gorter de Vries for critically reading the manuscript.

Supplementary material

Table S5: Primers used in this study

Primer	Sequence	Purpose
812	TCATGTAATTAGTTATGTCACGCTTAC ATTC	Amplification of p426-TEF-amdS backbone for Gibson Assembly
921	AAAACTTAGATTAGATTGCTATGCTTT CTTTCTAATGAGC	Amplification of p426-TEF-amdS backbone for Gibson Assembly
0491	GCTCATTAGAAAGAAAGCATAGCAATC TAATCTAAGTTTTAAAGTTTCGGTATA CTTAGCAGACAG	Amplification of Se*MALT1* from *S. eubayanus* CBS 12357 for Gibson Assembly
0492	GGAGGGCGTGAATGTAAGCGTGACATA ACTAATTACATGATACCCTAATCAAGT AAATAGATAATAAAGTTAATGTG	Amplification of Se*MALT1* from *S. eubayanus* CBS 12357 for Gibson Assembly
0632	GGAGGGCGTGAATGTAAGCGTGACATA ACTAATTACATGATGCGCTAAGAGTCA TCAAT	Amplification of Se*MALT2* from *S. eubayanus* CBS 12357 for Gibson Assembly
0633	GCTCATTAGAAAGAAAGCATAGCAATC TAATCTAAGTTTTGAGGCGTGATATGC TCCAT	Amplification of Se*MALT2* from *S. eubayanus* CBS 12357 for Gibson Assembly
0671	GGAGGGCGTGAATGTAAGCGTGACATA ACTAATTACATGATGTCAGATAACAAA ACCAGATACC	Amplification of Se*MALT3* from *S. eubayanus* CBS 12357 for Gibson Assembly
0672	GCTCATTAGAAAGAAAGCATAGCAATC TAATCTAAGTTTTCGATAGAATATCCT GCTGAACC	Amplification of Se*MALT3* from *S. eubayanus* CBS 12357 for Gibson Assembly
940	GCTCATTAGAAAGAAAGCATAGCAATC TAATCTAAGTTTTCAACGTACCGGGCT TGAGGGACATACAG	Amplification of *ScAGT1* from *S. cerevisiae* CEN.PK113-7D
941	GGAGGGCGTGAATGTAAGCGTGACATA ACTAATTACATGACTAGCTGAGGGTTT TGGGAGCAGTCAAAGG	Amplification of *ScAGT1* from *S. cerevisiae* CEN.PK113-7D
0178	GGGAACAAAAGCTGGAGCTCATAG	Out-Out diagnostic PCR of Gibson Assembly constructs
947	TACCGGCCGCAAATTAAAGC	Out-Out diagnostic PCR of Gibson Assembly constructs
0627	TTATAGGCGGGATGGGATGTTC	In-Out diagnostic PCR of Se*MALT1* Gibson Assembly construct
0628	CTGGCCTTCCAGACCGTTATAC	In-Out diagnostic PCR of Se*MALT2/4* Gibson Assembly construct
0629	TCTTCTAATGGTGGTCGCCTTC	In-Out diagnostic PCR of Se*MALT3* Gibson Assembly construct
036	TTTACAATATAGTGATAATCGTGGACT AGAGCAAGATTTCAAATAAGTAACAGC AGCAAACATAGCTTCAAAATGTTTCTA CTCCTTTTTTAC	Amplification of transporter gene expression cassette for construction of strains with transporter and Sc*MAL12*
039	CACCTTTCGAGAGGACGATGCCCGTGTC TAAATGATTCGACCAGCCTAAGAATGT TCAACGCCGCAAATTAAAGCCTTCG	Amplification of transporter gene expression cassette for construction of strains with transporter and Sc*MAL12*
596	TGTAAATATCTAGGAAATACACTTGTG TATACTTCTCGCTTTTCTTTTATTTTTT TTTGTAGTTTATCATTATCAATACTCG CCATTTC	Amplification of Sc*MAL12* expression cassette for construction of strains with transporter and Sc*MAL12*
355	TGTAAATATCTAGGAAATACACTTGTG TATACTTCTCGCTTTTCTTTTATTTTTT TTTGTAGTTTATCATTATCAATACTCG CCATTTC	Amplification of Sc*MAL12* expression cassette for construction of strains with transporter and Sc*MAL12*

095	GTGAGGTAAGTGTGCAAAGG	Out-Out diagnostic PCR of constructed guide RNA plasmids
219	GTCTAACTCCTTCCTTTTCGG	Out-Out diagnostic PCR of constructed guide RNA plasmids
226	ACTCGTACAAGGTGCTTTTAACTTG	Out-Out diagnostic PCR of integration in IMZ616 sga1
224	TTGATGTAAATATCTAGGAAATACACTTG	Out-Out diagnostic PCR of integration in IMZ616 sga1
1850	GAAGGGTCTATCTTCAATATTGAATAGAAAGAGAAACGAAAGCGATTCGATTTCCAGTAATTTAGCCTAGGTACTCTATGTTGGGCCATCATTGATTTACCCGAAACTGCCGGTAGAACT	*SeMALT1* repair (+) Deletion +63 - +1600
1851	AGTTCTACCGGCAGTTTCGGGTAAATCAATGATGGCCCAACATAGAGTACCTAGGCTAAATTACTGGAAATCGAATCGCTTTCGTTTCTCTTTCTATTCAATATTGAAGATAGACCCTTC	*SeMALT1* repair (-) Deletion +63 -+1600
1328	ATGAAGGGTCTATCCTCAATGATAAATAGAAAGAAGTGCAACGGTAACTCGAGCTCAATAGAAGATATGAAAGCTTCCGCCGAGGAGAGAGAGCAAAGCACCCCATCTCTAATGGATTGA	*SeMALT2/T4* repair (+) Deletion +60 - +1782
1329	TCAATCCATTAGAGATGGGGTGCTTTGCTCTCTCTCCTCGGCGGAAGCTTTCATATCTTCTATTGAGCTCGAGTTACCGTTGCACTTCTTTCTATTTATCATTGAGGATAGACCCTTCAT	*SeMALT2/T4* repair (-) Deletion +60 - +1782
1330	ATGAAGGGCTTATCCTCACTGATAAACAGAAAAAAAAAACAAGATTGACTCTAATTCAAATGATATGGAAACTTCCATGGTGGAAGAAGGGCGAAGCACACCATCTATTACGAATTTATGA	*SeMALT3* repair (+) Deletion +60 - +1785
1331	TCATAAATTCGTAATAGATGGTGTGCTTCGCCCTTCTTCCACCATGGAAGTTTCCATATCATTTGAATTAGAGTCAATCTTGTTTTTTTTTCTGTTTATCAGTGAGGATAAGCCCTTCAT	*SeMALT3* repair (-) Deletion +60 - +1785
1671	AGGTTCCTGGGCAGTGAAGC	*SeMALT1* deletion check
1672	AGGTCCAAGTCCTCTGTAAG	*SeMALT1* deletion check
1673	CCCTGAATGATCTGGTGAAC	*SeMALT*2/T4 deletion check
1674	AACACCCGCTATATTCCTCG	*SeMALT*2/T4 deletion check
1675	CATAGCTGGTACAGGATACG	*SeMALT3* deletion check
1676	GCCGTGAATAGCTTAAGGTG	*SeMALT3* deletion check
043	CGAGCAAATGCCTGCAAATCG	Transporter integration check Out-In
42	AGCAGCAAACAGCGTCTTGTC	Transporter integration check In-Out

Table S6: List of genes added or modified in the new *S. eubayanus* CBS 12357T reference genome and annotations. The descriptions were based on similarity with *S. cerevisiae* orthologs.

				Similarity with *S. cerevisiae* gene		
				Systematic		
CHR	Start	End	Length	Name	Name	Description
*Se*CHRI	5572	6660	1089	YJR153W	*PGU1*	Endo-polygalacturonase
*Se*CHRI	9928	14679	4752	YAL063C	*FLO9*	Flo1p homolog
*Se*CHRI	10391	13801	3411	YAL064W-B	YAL064W-B	Fungal-specific protein of unknown function
*Se*CHRI	177093	179285	2193	YAR035W	*YAT1*	Outer carnitine acetyltransferase, mitochondrial
*Se*CHRI	187599	193115	5517	YAR050W	*FLO1*	FLO1 putative cell wall glycoprotein
*Se*CHRI	194550	195545	996	YJL213W	YJL213W	Similarity to *Methanobacterium* aryldialkylphosphatase related protein
*Se*CHRI	197031	197744	714	YDR533C	*HSP31*	Strong similarity to hypothetical proteins YPL280w, YOR391c and YMR322c
*Se*CHRI	199000	199770	771	YKL071W	YKL071W	Weak similarity to *A. parasiticus* nor-1 protein
*Se*CHRI	202624	204504	1881	YIL169C	YIL169C	Similarity to glucan 1,4-alpha-glucosidase Sta1p and YAR066w
*Se*CHRII	10694	11368	675	YFL060C	*SNO3*	Proximal ORF, stationary phase induced gene
*Se*CHRII	11798	12739	942	YFL059W	*SNZ3*	Snooze: stationary phase-induced gene family
*Se*CHRII	13335	14357	1023	YDL244W	*THI13*	Thiamine biosynthetic enzyme
*Se*CHRII	187048	194272	7224	YBL017C	*PEP1*	Carboxypeptidase Y sorting receptor in late Golgi
*Se*CHRII	204314	205498	1185	YBL013W	*FMT1*	Probable met-tRNA formyltransferase, mitochondrial
*Se*CHRII	553563	564431	10869	YDR150W	*NUM1*	Protein with variable number of tandem repeats of a 64 amino-acid polypeptide
*Se*CHRII	870964	871440	477	YDR319C	*YFT2*	Member of the highly conserved FIT family of proteins involved in triglyceride droplet biosynthesis
*Se*CHRII	920553	922265	1713	YDR342C	*HXT7*	Hexose transporter
*Se*CHRII	925500	930623	5124	YDR345C	*HXT3*	Low-affinity glucose transporter
*Se*CHRII	1067774	1072780	5007	YDR420W	*HKR1*	Type 1 membrane protein with EF hand motif
*Se*CHRIII	264091	270210	6120	YCR089W	*FIG2*	predicted GPI-anchored cell wall protein

*Se*CHRIV	38153	39994	1842	YDL229W	*SSB1*	Heat shock protein of HSP70 family
*Se*CHRIV	116188	117552	1365	YDL189W	*RBS1*	Hypothetical protein
*Se*CHRIV	117842	118978	1137	YDL188C	*PPH22*	Serine-threonine protein phosphatase 2A
*Se*CHRIV	220021	221343	1323	YDL131W	*LYS21*	Homocitrate synthase, highly homologous to YDL182W
*Se*CHRIV	373695	377804	4110	YDL037C	*BSC1*	Strong similarity to glucan 1,4-alpha-glucosidase
*Se*CHRIV	599827	601230	1404	YBR092C	*PHO3*	Acid phosphatase, constitutive
*Se*CHRIV	601685	603088	1404	YBR092C	*PHO3*	Acid phosphatase, constitutive
*Se*CHRIV	853943	855004	1062	YBR231C	*SWC5*	Similarity to human p97 homologous protein
*Se*CHRV	26219	27724	1506	YNR073C	YNR073C	Strong similarity to *E.coli* D-mannonate oxidoreductase, identical to YEL070w
*Se*CHRV	28195	29916	1722	YEL069C	*HXT13*	Hexose transporter
*Se*CHRV	195052	198240	3189	YER017C	*AFG3*	ATP-dependent metalloprotease
*Se*CHRV	577191	577442	252	YHL047C	*ARN2*	Similarity to *C. carbonum* toxin pump
*Se*CHRVI	3434	6913	3480	YAL063C	*FLO9*	Flo1p homolog
*Se*CHRVI	192972	193928	957	YFR034C	*PHO4*	Myc-type helix-loop-helix transcription factor
*Se*CHRVI	228124	238437	10314	YDR134C	YDR134C	Strong similarity to Flo1p
*Se*CHRVI	256654	257742	1089	YJR153W	*PGU1*	Endo-polygalacturonase
*Se*CHRVII	6946	8559	1614	YIR039C	YPS6	GPI-anchored aspartic protease
*Se*CHRVII	606893	607519	627	YPR071W	YPR071W	Strong similarity to YIL029c
*Se*CHRVIII	26976	29960	2985	YDR534C	*FIT1*	Similarity to YOR383c,Sta1p and pig mucin
*Se*CHRVIII	253415	254362	948	YOR083W	*WHI5*	Weak similarity to YKR091w
*Se*CHRIX	4707	5837	1131	YNL134C	YNL134C	Similarity to C. carbonum *toxD* gene
*Se*CHRIX	6568	8187	1620	YIR039C	*YPS6*	GPI-anchored aspartic protease
*Se*CHRIX	8612	9748	1137	YFL062W	*COS4*	Protein with strong similarity to subtelomerically-encoded proteins s
*Se*CHRIX	29310	30941	1632	YIL159W	*BNR1*	Bni1p-related protein, helps regulate reorganization of the actin cytoskeleton
*Se*CHRIX	238441	239202	762	YIL049W	*DFG10*	Protein involved in filamentous growth, cell

						polarity, and cellular elongation
*Se*CHRIX	365797	369945	4149	YIR019C	*MUC1*	Cell surface flocculin with structure similar to serine threonine-rich GPI-anchored cell wall proteins
*Se*CHRIX	371169	371381	213	YIR020W-A	YIR020W-A	Dubious open reading frame
*Se*CHRX	3742	5622	1881	YIL169C	YIL169C	Similarity to glucan 1,4-alpha-glucosidase Sta1p and YAR066w
*Se*CHRX	8474	9253	780	YKL071W	YKL071W	Weak similarity to *A. parasiticus* nor-1 protein
*Se*CHRX	10614	11939	1326	YJL213W	YJL213W	Similarity to *Methanobacterium* aryldialkylphosphatase related protein
*Se*CHRX	13383	17423	4041	YAL063C	*FLO9*	Flo1p homolog
*Se*CHRX	23126	24451	1326	YJL213W	YJL213W	Similarity to *Methanobacterium* aryldialkylphosphatase related protein
*Se*CHRX	102189	103517	1329	YJL164C	*TPK1*	Putative catalytic subunit of cAMP-dependent protein kinase
*Se*CHRX	145708	146898	1191	YKR059W	*TIF1*	Translation initiation factor eIF4A
*Se*CHRX	287825	288391	567	YJL075C	*APQ13*	questionable ORF
*Se*CHRX	348364	349281	918	YJL043W	YJL043W	Similarity to hypothetical protein YKR015c
*Se*CHRX	432474	434615	2142	YJR004C	*SAG1*	Alpha-agglutinin
*Se*CHRX	702344	705556	3213	YJR150C	*DAN1*	Protein induced during anaerobic growth
*Se*CHRX	732395	734113	1719	YEL069C	*HXT13*	Hexose transporter
*Se*CHRX	734531	736036	1506	YNR073C	YNR073C	Strong similarity to *E. coli* D-mannonate oxidoreductase, identical to YEL070w
*Se*CHRX	743018	744151	1134	YFL062W	*COS4*	Protein with strong similarity to subtelomerically-encoded proteins
*Se*CHRX	744577	746190	1614	YIR039C	*YPS6*	GPI-anchored aspartic protease
*Se*CHRXI	54859	56775	1917	YKL198C	*PTK1*	Probable serine threonine-specific protein kinase (EC 2.7.1.-)
*Se*CHRXI	545410	545940	531	YKR062W	*TFA2*	Small subunit of TFIIE transcription factor
*Se*CHRXI	635126	639253	4128	YKR102W	*FLO10*	Protein with similarity to flocculation protein Flo1p
*Se*CHRXI	29343	32663	3321	YAL063C	*FLO9*	Putative Flo1p homolog
*Se*CHRXI	463503	464000	498	YLR154C-H	YLR154C	Hypothetical protein
*Se*CHRXI	519195	527990	8796	YJL084C	*ART3*	Similarity to hypothetical protein YKR021w

*Se*CHRXI	561132	570266	9135	YJL084C	*ART3*	Similarity to hypothetical protein YKR021w
*Se*CHRXI	644658	644999	342	YLR204W	*QRI5*	Protein of unknown function
*Se*CHRXI	661072	663138	2067	YLR214W	*FRE1*	Ferric (and cupric) reductase
*Se*CHRXI	664035	666101	2067	YLR214W	*FRE1*	Ferric (and cupric) reductase
*Se*CHRXI	1089672	1090070	399	YLR437C	*DIF1*	Protein that regulates nuclear localization of Rnr2p and Rnr4p
*Se*CHRXII	277902	278318	417	YMR003W	*AIM34*	Hypothetical protein
*Se*CHRXII	601432	603129	1698	YMR173W	*DDR48*	Flocculent specific protein
*Se*CHRXII	904661	907945	3285	YMR317W	YMR317W	Similarity to mucins, glucan 1,4-alpha-glucosidase and exo-alpha-sialidase
*Se*CHRXII	916196	917173	978	YPL273W	*SAM4*	Strong similarity to hypothetical protein YLL062c
*Se*CHRXII	917562	919325	1764	YPL274W	*SAM3*	Strong similarity to amino-acid transport proteins
*Se*CHRXII	923767	925488	1722	YEL069C	*HXT13*	Hexose transporter
*Se*CHRXII	925958	927463	1506	YNR073C	YNR073C	Strong similarity to *E. coli* D-mannonate oxidoreductase, identical to YEL070w
*Se*CHRXII	928789	929463	675	YFL060C	*SNO3*	*SNZ2* proximal ORF, stationary phase induced gene
*Se*CHRXII	929892	930788	897	YFL059W	*SNZ3*	Snooze: stationary phase-induced gene family
*Se*CHRXII	931430	932452	1023	YDL244W	*THI13*	Thiamine biosynthetic enzyme
*Se*CHRXV	19114	22533	3420	YNL327W	*EGT2*	Cell-cycle regulation protein
*Se*CHRXV	128069	129781	1713	YNL270C	*ALP1*	Protein highly homologous to permeases Can1p and Lyp1p for basic amino acids
*Se*CHRXV	130734	132569	1836	YNL268W	*LYP1*	Lysine permease
*Se*CHRXV	190720	192210	1491	YNL240C	*NAR1*	Strong similarity to *K. marxianus* LET1 protein
*Se*CHRXV	549110	556552	7443	YBL017C	*PEP1*	Carboxypeptidase Y sorting receptor in late Golgi
*Se*CHRXV	641192	642412	1221	YNR012W	*URK1*	Uridine kinase
*Se*CHRXV	697011	699341	2331	YNR044W	*AGA1*	Anchorage subunit of a-agglutinin
*Se*CHRXV	732884	734602	1719	YEL069C	*HXT13*	Hexose transporter
*Se*CHRXV	735045	736550	1506	YNR073C	YNR073C	Strong similarity to E.coli D-mannonate

						oxidoreductase, identical to YEL070w
*Se*CHRX VI	2599	2961	363	YOL161C	*PAU20*	Strong similarity to members of the Srp1p/Tip1p family
*Se*CHRX VI	4738	6558	1821	YOL158C	*ENB1*	Similarity to subtelomeric encoded proteins
*Se*CHRX VI	7603	7851	249	YHL047C	*ARN2*	Similarity to *C. carbonum* toxin pump
*Se*CHRX VI	7895	9664	1770	YGR287C	*IMA1*	Strong similarity to maltase
*Se*CHRX VI	11123	12841	1719	YJL219W	*HXT9*	High-affinity hexose transporter
*Se*CHRX VI	13815	15572	1758	YBR299W	*MAL32*	Maltase (EC 3.2.1.20)
*Se*CHRX VI	16413	18254	1842	YBR298C	*MAL31*	Maltose permease
*Se*CHRX VI	21820	23589	1770	YGR287C	*IMA1*	Strong similarity to maltase
*Se*CHRX VI	907607	909334	1728	YKR105C	*VBA5*	Strong similarity to Sge1p and hypothetical protein YCL069w

Table S7: Genes with a |fold-difference| in expression level ≥ 4 in glucose and maltose-grown cultures of *S. eubayanus* CBS 12357^T. Data represent average expression intensity and mean deviation of two replicate genome-wide transcript datasets.

			Similarity to *S.cerevisiae* gene			Glucose	Maltose	Fold	Adjusted
CHR	Start	Stop	Name	Syst name	description	FPKM±Dev	FPKM±Dev	change	*p*-value
*Se*CHRXVI	13815	15572	*MAL32*	*YBR299W*	Maltase (EC 3.2.1.20)	9.9 ±0.2	5218.4 ± 365.5	525.6	0
*Se*CHRV	569448	571205	*MAL32*	*YBR299W*	Maltase (EC 3.2.1.20)	9.6 ± 0.0	4620.4 ± 384.1	481.3	0
*Se*CHRV	566765	568606	*MAL31*	*YBR298C*	Maltose permease (*SeMALT2*)	5.5 ± 0.4	1451.0 ± 39.7	262.7	0
*Se*CHRXVI	16413	18254	*MAL31*	*YBR298C*	Maltose permease (*SeMALT4*)	6.9 ± 0.4	1683.8 ± 30.7	244.5	0
*Se*CHRXIII	4195	5964	*IMA1*	*YGR287C*	Major isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase)	8.4 ± 1.2	1671.4 ± 283.7	199.9	0
*Se*CHRXVI	21821	23590	*IMA1*	*YGR287C*	Major isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase)	3.4 ± 0.7	645.2 ± 95.7	189.9	0
*Se*CHRIV	971754	973529	*DAK2*	*YFL053W*	Dihydroxyacetone kinase	19.2 ± 0.6	3342.3 ± 171.5	174.1	0
*Se*CHRII	8497	10338	*MAL31*	*YBR298C*	Maltose permease (*SeMALT1*)	0.5 ± 0.0	30.8 ± 1.5	67.7	3.18E-82
*Se*CHRXVI	25036	25626	YJL218W	YJL218W	Putative acetyltrasferase	8.3 ± 2.3	528.3 ± 4.3	63.9	2.10E-246
*Se*CHRIV	968740	970695	*AQY3*	*YFL054C*	Putative channel-like protein; similar to Fps1	10.7 ± 2.2	529.6 ± 22.0	49.3	0
*Se*CHRII	5766	7535	*IMA1*	*YGR287C*	Major isomaltase (alpha-1,6-glucosidase/alpha-methylglucosidase)	1.6 ± 0.1	66.6 ± 5.1	41.7	2.65E-150
*Se*CHRIV	974654	975250	*REE1*	*YJL217W*	Cytoplasmic protein involved in the regulation of enolase	27.0 ± 1.1	1079.8 ± 76.4	40.0	2.34E-293
*Se*CHRX	326579	327577	*TDH1*	*YJL052W*	Glyceraldehyde-3-phosphate dehydrogenase 1	39.1 ± 0.1	1196.1 ± 318.7	30.6	8.19E-188
*Se*CHRV	564762	565970	*MAL33*	*YBR297W*	MAL-activator protein; part of complex locus MAL3	10.0 ± 1.2	196.3 ± 17.0	19.6	4.38E-171
*Se*CHRIV	512918	513952	*YRO2*	*YBR054W*	Homolog to HSP30 heat shock protein YRO1	128.1 ± 6.9	2348.5 ± 110.4	18.3	2.46E-238
*Se*CHRXV	735045	736550	YNR073C	YNR073C	Mannitol dehydrogenase	1.8 ± 0.2	31.8 ± 8.3	17.6	8.08E-62
*Se*CHRX	732395	734113	*HXT13*	*YEL069C*	Hexose transporter	14.2 ± 3.8	202.2 ± 19.0	14.3	3.41E-113
*Se*CHRIV	552424	553068	*HSP26*	*YBR072W*	Heat shock protein of 26 kDa, expressed in entry to stationary phase	137.5 ± 19.5	1520.3 ± 33.7	11.1	1.22E-147
*Se*CHRVII	585691	587421	*MUP1*	*YGR055W*	High affinity methionine permease	5.1 ± 1.6	55.2 ± 7.4	10.8	9.30E-67
*Se*CHRVII	934199	934486	*SPG1*	*YGR236C*	Questionable ORF	6.3 ± 1.6	65.3 ± 5.5	10.3	2.33E-31
*Se*CHRXIII	942285	943424	*FDH1*	*YOR388C*	Protein with similarity to formate dehydrogenases	5.9 ± 0.6	53.3 ± 3.1	9.1	2.94E-64

*Se*CHRXVI	829072	830475	*TDA6*	*YPR157W*	Putative vacuolar protein sorting-associated	7.9 ± 0.0	71.2 ± 4.8	9.0	3.39E-86
*Se*CHRX	727746	728780	YNR071C	YNR071C	Uncharacterized isomerase	90.7 ± 1.1	814.2 ± 27.6	9.0	5.25E-145
*Se*CHRVII	23171	24301	YNL134C	YNL134C	Uncharacterized protein	15.7 ± 4.2	131.7 ± 20.3	8.4	2.61E-64
*Se*CHRV	81046	82548	*YEF1*	*YEL041W*	ATP-NADH kinase; phosphorylates both NAD and NADH	28.6 ± 1.9	233.5 ± 15.0	8.2	1.32E-114
*Se*CHRXIII	669178	670125	YMR206W	YMR206W	Uncharacterized protein	2.2 ± 0.3	17.0 ± 6.7	7.8	2.05E-19
*Se*CHRXIV	330291	331349	*YGP1*	*YNL160W*	Secreted glycoprotein produced in response to nutrient limitation	745.5 ± 120.3	5588.8 ±487.2	7.5	6.62E-94
*Se*CHRV	468857	469288	*SPI1*	*YER150W*	Strong similarity to putative cell surface glycoprotein Sed1p	233.5 ± 74.6	1727.5 ±163.2	7.4	1.79E-58
*Se*CHRXIV	685860	686156	YNR034W-A	YNR034W-A	Uncharacterized protein	1188.2 ± 537.1	8530.4 ± 862.4	7.2	1.04E-09
*Se*CHRV	264185	265087	*PIC2*	*YER053C*	Mitochondrial copper and phosphate carrier	129.1 ± 24.2	920.6 ± 57.0	7.1	4.21E-84
*Se*CHRXIII	9572	11416	*MAL31*	*YBR298C*	Maltose permease (*SeMALT3*)	29.9 ± 1.1	204.6 ± 30.1	6.8	5.22E-84
*Se*CHRXIII	935354	937039	*STL1*	*YDR536W*	Sugar transporter-like protein	4.0 ± 1.2	26.6 ± 1.2	6.6	7.96E-40
*Se*CHRXI	300992	301213	YKL065W-A	YKL065W-A	Uncharacterized protein	7.3 ± 3.0	48.0 ± 4.9	6.6	1.94E-15
*Se*CHRVII	1050057	1051673	*YPS6*	*YIR039C*	GPI-anchored aspartic protease	2.7 ± 0.8	17.5 ± 3.6	6.5	1.92E-27
*Se*CHRXIV	365428	366558	YNL134C	YNL134C	NADH-dependent aldehyde reductase	68.1 ± 11.1	440.1 ±52.0	6.5	3.99E-75
*Se*CHRV	298146	298631	*RGI1*	*YER067W*	Protein of unknown function; involved in energy metabolism	769.0 ± 77.4	4755.0 ± 13.1	6.2	1.39E-99
*Se*CHRII	353374	355188	*BAP3*	*YDR046C*	Valine transporter	7.9 ± 0.3	48.1 ± 0.2	6.1	3.65E-62
*Se*CHRII	326147	327121	*MRH1*	*YDR033W*	Strong similarity to putative heat shock protein YRO2	541.4 ± 26.6	3171.9 ±283.0	5.9	7.70E-81
*Se*CHRX	8474	9253	YKL071W	YKL071W	Putative protein of unknown function	6.1 ± 0.7	33.0 ± 3.0	5.4	4.48E-27
*Se*CHRXVI	223023	224216	*OYE3*	*YPL171C*	NAD(P)H dehydrogenase	39.1 ± 1.3	210.0 ± 15.0	5.4	1.21E-72
*Se*CHRIII	151700	152689	*HSP30*	*YCR021C*	Heat shock protein located in the plasma membrane	23.1 ± 3.9	122.2 ± 5.3	5.3	8.78E-53
*Se*CHRVIII	324960	325898	*GCY1*	*YOR120W*	Similar to mammalian aldo keto reductases	192.6 ± 73.2	995.6 ± 14.7	5.2	2.35E-35
*Se*CHRXIII	373028	373924	YLL056C	YLL056C	Putative protein of unknown function	21.8 ± 4.8	111.5 ± 13.4	5.1	1.52E-40
*Se*CHRI	199000	199770	YKL071W	YKL071W	Putative protein of unknown function	11.4 ± 0.7	56.9 ± 5.9	5.0	2.18E-34
*Se*CHRII	488814	489014	*COX26*	*YDR119W-A*	Stabilizes or regulates formation of respiratory chain complexes	46.7 ± 5.3	228.4 ± 13.9	4.9	8.40E-34
*Se*CHRI	60828	61628	YAL037W	YAL037W	Putative protein of unknown function	5.4 ± 1.1	26.1 ± 0.1	4.8	1.01E-20
*Se*CHRXIII	923767	925488	*HXT13*	*YEL069C*	Putative transmembrane polyol transporter	61.6 ± 0.7	294.3 ± 33.3	4.8	4.58E-63
*Se*CHRX	594900	595748	YJR096W	YJR096W	Xylose and arabinose reductase	34.4 ± 6.4	162.8 ± 8.6	4.7	6.20E-46
*Se*CHRXI	567594	568697	*ECM4*	*YKR076W*	ExtraCellular Mutant	33.5 ± 6.2	157.1 ± 7.3	4.7	9.69E-48
*Se*CHRVIII	434753	437188	*GAC1*	*YOR178C*	Regulatory subunit for Glc7p	14.5 ± 1.2	67.2 ± 5.7	4.6	7.52E-54

*Se*CHRXVI	897255	897722	*HPA2*	*YPR193C*	Histone and other Protein Acetyltransferase	17.7± 1.8	81.3 ± 11.3	4.6	7.43E-28
*Se*CHRXII	807640	808032	YLR297W	YLR297W	Protein of unknown function	71.3 ± 4.3	326.6 ± 0.9	4.6	1.67E-50
*Se*CHRII	127205	127621	*MOH1*	*YBL049W*	Protein of unknown function, essential for stationary phase survival	31.6± 1.8	143.0 ± 1.0	4.5	4.00E-38
*Se*CHRVII	289727	290107	*GPG1*	*YGL121C*	Proposed gamma subunit of the heterotrimeric G protein	293.9 ± 26.0	1275.5 ±173.3	4.3	3.89E-48
*Se*CHRXVI	625902	629342	*CSR2*	*YPR030W*	Similarity to YBL101c	11.6 ± 1.5	50.1 ± 5.3	4.3	7.74E-46
*Se*CHRIV	542614	543234	*TIP1*	*YBR067C*	Cell wall mannoprotein	131.0 ± 33.5	567.5 ± 138.0	4.3	1.09E-26
*Se*CHRV	455522	456415	*MAG1*	*YER142C*	3-methyladenine DNA glycosylase	14.3 ± 1.0	61.9 ± 7.2	4.3	2.97E-32
*Se*CHRVII	949163	949600	*FMP43*	*YGR243W*	Highly conserved subunit of the mitochondrial pyruvate carrier	15.4 ± 2.3	66.5 ± 1.1	4.3	2.48E-24
*Se*CHRXI	8566	10413	*JEN1*	*YKL217W*	Carboxylic acid transporter protein homolog	5.2 ± 0.6	22.0 ± 0.1	4.3	1.70E-29
*Se*CHRVII	10469	11500	YNR071C	YNR071C	Aldose 1-epimerase	485.0 ± 46	2021.5 ± 30.5	4.2	7.63E-62
*Se*CHRIX	397417	398181	*NRE1*	*YIR035C*	Putative cytoplasmic short-chain dehydrogenase reductase	154.4 ± 11.9	642.0 ± 48.6	4.2	4.42E-51
*Se*CHRIV	706015	707070	*ADH5*	*YBR145W*	Alcohol dehydrogenase isoenzyme V	75.6 ± 0.3	312.9 ± 15.6	4.1	5.42E-57
*Se*CHRXIII	200932	202473	*RRN11*	*YML043C*	Component of rDNA transcription factor CF	20.2 ± 1.6	5.0 ± 0.5	-4.0	1.56E-21
*Se*CHRXV	116156	119050	*MSH2*	*YOL090W*	MutS homolog encoding major mismatch repair activity	22.2 ± 3.4	5.5 ± 0.6	-4.0	6.56E-26
*Se*CHRII	232378	232776	*HTA1*	*YDR225W*	Histone H2A (HTA1 and HTA2 code for nearly identical proteins)	6216.1 ± 95.8	1543.9 ±188.1	-4.0	1.25E-45
*Se*CHRX	68803	71355	*SWE1*	*YJL187C*	Protein kinase homolog	22.6 ± 1.2	5.6 ± 1.1	-4.0	1.12E-26
*Se*CHRXVI	305949	306761	*HHO1*	*YPL127C*	Histone H1	136.2 ± 7.3	33.6 ± 5.4	-4.1	1.57E-35
*Se*CHRXII	38986	39858	*AQY1*	*YPR192W*	Similarity to plasma membrane and water channel proteins	54.1 ± 14.2	13.3 ± 1.5	-4.1	6.38E-21
*Se*CHRXIV	714569	716618	*NOG2*	*YNR053C*	Strong similarity to human breast tumor associated autoantigen	96.0 ± 14.2	23.0 ± 3.1	-4.2	3.41E-34
*Se*CHRXI	164418	165815	*LTV1*	*YKL143W*	Low temperature viability protein	28.9 ± 4.9	6.9 ± 0.3	-4.2	5.77E-23
*Se*CHRXIV	568431	568742	*HHF1*	*YBR009C*	Histone H4; core histone protein required for chromatin assembly	6315.8 ± 13.1	1489.5 ± 91.2	-4.2	3.61E-58
*Se*CHRXIII	875200	876345	*SCW10*	*YMR305C*	Member of the glucanase gene family	209.0 ±25.4	48.6 ± 6.7	-4.3	1.61E-38
*Se*CHRII	519002	519394	*CCW22*	*YDR134C*	Cell wall protein; YDR134C has a paralog,	4393.2 ±171.7	1003.3 ± 25.8	-4.4	4.41E-65
*Se*CHRVII	850827	852332	*CRH1*	*YGR189C*	Similarity to *Aspergillus fumigatus* rAsp	475.8 ± 41.7	108.6 ± 6.6	-4.4	4.86E-52

*Se*CHRXII	657361	658839	*TUB4*	*YLR212C*	Gamma tubulin-like protein, interacts with Spc98p and Spc97p	44.5 ± 1.7	10.1 ± 1.7	-4.4	1.19E-32
*Se*CHRIII	185259	186302	*FEN1*	*YCR034W*	Probable subunit of 1,3-beta-glucan synthase\; homolog of ELO1	377.5 ± 20.0	85.3 ± 10.5	-4.4	2.04E-46
*Se*CHRXI	249340	250086	*CWP1*	*YKL096W*	Cell wall mannoprotein	1334.2 ± 125.2	299.6 ± 33.9	-4.5	3.55E-55
*Se*CHRXIV	380345	382882	*SPC98*	*YNL126W*	Spindle pole body component	20.1 ± 0.2	4.5 ± 0.6	-4.5	4.10E-16
*Se*CHRII	153948	156062	*POL12*	*YBL035C*	B subunit of DNA polymerase alpha-primase complex	18.1 ± 4.4	4.0 ± 0.1	-4.5	3.34E-21
*Se*CHRIX	113174	114583	*SIM1*	*YIL123W*	Involved in cell cycle regulation and aging	287.9 ± 23.8	63.7 ± 7.6	-4.5	1.55E-48
*Se*CHRXII	431462	432901	*PUT1*	*YLR142W*	Proline oxidase (proline dehydrogenase)	19.0 ± 2.6	4.2 ± 1.2	-4.5	1.27E-19
*Se*CHRXV	416676	417626	*GIC1*	*YHR061C*	GTPase-interacting component 1	61.3 ± 3.4	13.5 ± 0.6	-4.5	6.35E-36
*Se*CHRIX	281450	284902	*IRR1*	*YIL026C*	Subunit of the cohesin complex	18.3 ± 3.7	4.0 ± 0.1	-4.6	4.53E-28
*Se*CHRV	216592	217746	*MIG3*	*YER028C*	Transcriptional regulator	32.4 ± 0.1	7.1 ± 0.2	-4.6	4.56E-29
*Se*CHRXIII	690119	691672	*GAS3*	*YMR215W*	Similarity to GAS1 protein	84.6 ± 7.6	18.4 ± 0.3	-4.6	3.51E-49
*Se*CHRV	78890	80452	*GDA1*	*YEL042W*	Guanosine diphosphatase located in the Golgi	125.7 ± 3.4	27.0 ± 1.6	-4.7	8.91E-55
*Se*CHRVIII	723692	724654	YOR342C	YOR342C	Protein of unknown function	76.3 ± 14.1	16.0 ± 0.9	-4.8	6.75E-32
*Se*CHRXI	338942	340531	*PRI2*	*YKL045W*	p58 polypeptide of DNA primase	24.6 ± 3.6	5.2 ± 1.1	-4.8	1.36E-24
*Se*CHRIV	356746	357555	*KNH1*	*YDL049C*	KRE9 homolog	44.9 ± 2.5	9.4 ± 1.2	-4.8	2.34E-28
*Se*CHRII	478219	479349	*PDS1*	*YDR113C*	42-kDa nuclear protein	57.9 ± 2.9	12.1 ± 0.1	-4.8	1.49E-38
*Se*CHRVIII	737587	739464	*PUT4*	*YOR348C*	Putative proline-specific permease	16.3 ± 5.7	3.4 ± 0.5	-4.8	2.18E-18
*Se*CHRXIII	225859	227010	*YOX1*	*YML027W*	Homeobox-domain containing protein	67.2 ± 7.6	13.9 ± 1.0	-4.8	2.01E-40
*Se*CHRXV	257246	258829	YOL019W	YOL019W	Protein of unknown function	53.1 ± 0.5	10.9 ± 0.3	-4.9	2.32E-45
*Se*CHRIX	70415	72874	*AXL2*	*YIL140W*	Localizes to the plasma membrane	25.0 ± 3.8	5.1 ± 0.2	-4.9	2.83E-33
*Se*CHRIV	272749	274293	*DUN1*	*YDL101C*	Protein kinase	37.2 ± 2.2	7.5 ± 0.7	-4.9	7.02E-37
*Se*CHRVII	441348	441710	*CGR1*	*YGL029W*	Weak similarity to human chromatin assembly factor I p150 chain	234.1 ± 43.3	46.9 ± 1.9	-5.0	6.22E-35
*Se*CHRXIV	707536	708876	*LYS9*	*YNR050C*	Saccharopine dehydrogenase (NADP+, L-glutamate forming)	262.6 ± 6.6	50.6 ± 2.2	-5.2	1.52E-72
*Se*CHRXI	236917	241500	*HSL1*	*YKL101W*	Putative protein kinase homologous to S. pombe cdr1 nim1	24.6 ± 4.8	4.7 ± 1.0	-5.2	9.62E-34
*Se*CHRXIII	545844	546851	*FDO1*	*YMR144W*	Protein involved in directionality of mating type switching	33.0 ± 5.3	6.3 ± 0.7	-5.2	3.51E-26
*Se*CHRXVI	364893	366263	*EEB1*	*YPL095C*	Acyl-coenzymeA:ethanol O-acyltransferase	132.0 ± 6.5	25.1 ± 0.7	-5.3	1.03E-60
*Se*CHRIV	597086	597862	*POL30*	*YBR088C*	Profilerating cell nuclear antigen (PCNA)	257.7 ± 24.3	48.0 ± 2.9	-5.4	2.48E-39
*Se*CHRX	48344	50713	*ACO2*	*YJL200C*	Putative mitochondrial aconitase isozyme	115.6 ± 6.0	21.3 ± 3.1	-5.4	1.56E-58
*Se*CHRX	463176	465695	*RBH2*	*YJR030C*	Putative protein of unknown function	19.5 ± 3.3	3.6 ± 0.6	-5.4	2.75E-31

*Se*CHRVI	246194	247399	*IRC7*	*YFR055W*	Beta-lyase involved in the production of thiols	38.6 ± 4.2	7.1 ± 0.7	-5.4	1.46E-34
*Se*CHRII	233489	233884	*HTB2*	*YBL002W*	Histone H2B (HTB1 and HTB2 code for nearly identical proteins)	5683.3 ± 69.9	1042.7 ±133.2	-5.5	1.39E-66
*Se*CHRVIII	240512	241426	*CDC21*	*YOR074C*	Thymidylate synthase	54.8 ± 9.2	10.0 ± 0.9	-5.5	7.46E-33
*Se*CHRXVI	238727	239494	*SVS1*	*YPL163C*	Serine and threonine rich protein.	158.6 ± 18.0	28.5 ± 7.3	-5.6	2.70E-39
*Se*CHRXIV	406937	409589	*DBP2*	*YNL112W*	TP-dependent RNA helicase of DEAD box family	84.8 ± 24.1	14.8 ± 2.4	-5.7	1.02E-35
*Se*CHRV	305067	307733	*RNR1*	*YER070W*	Ribonucleotide reductase	177.1 ± 51.7	30.8 ± 7.7	-5.8	4.36E-09
*Se*CHRXII	955392	956429	*SUR4*	*YLR372W*	Required for conversion of 24-carbon fatty acids to 26-carbon species	387.3 ± 53.3	65.7 ± 2.1	-5.9	1.30E-62
*Se*CHRXVI	62522	64180	*CLN2*	*YPL256C*	G(sub)1 cyclin	46.3 ± 8.4	7.3 ± 1.0	-6.3	1.10E-41
*Se*CHRXIII	917562	919325	*SAM3*	*YPL274W*	Strong similarity to amino-acid transport proteins	21.3 ± 0.0	3.4 ± 0.2	-6.3	1.04E-38
*Se*CHRXI	500540	502345	*GAP1*	*YKR039W*	General amino acid permease, proton symport transporter	773.1± 88.6	121.7 ± 4.2	-6.4	6.16E-92
*Se*CHRXIV	83384	84223	*PCL1*	*YNL289W*	G(sub)1 cyclin that associates with PHO85	278.8 ± 4.8	41.8 ± 2.8	-6.7	9.08E-86
*Se*CHRXV	280167	281201	*CSI2*	*YOL007C*	Structural component of the chitin synthase 3 complex	116.9 ± 11.8	16.1 ± 1.3	-7.3	9.17E-66
*Se*CHRXI	212836	213984	*RAD27*	*YKL113C*	42 kDa 5′ to 3′ exonuclease required for Okazaki fragment processing	65.1 ± 8.9	8.9 ± 0.9	-7.3	3.20E-52
*Se*CHRXII	612599	614092	*TOS4*	*YLR183C*	Similarity to YDR501w	61.9 ± 8.5	8.0 ± 0.3	-7.7	4.04E-60
*Se*CHRVIII	196815	198143	*STD1*	*YOR047C*	Dosage-dependent modulator of glucose repression	45.5 ± 0.4	5.8 ± 1.2	-7.8	1.10E-51
*Se*CHRIV	441107	442810	*MCD1*	*YDL003W*	Mitotic omosome Determinant; similar to *S. pombe RAD21*	61.2 ± 14.1	7.5 ± 1.0	-8.2	1.54E-51
*Se*CHRXIV	61224	61532	*TOS6*	*YNL300W*	Hypothetical protein	756.5 ± 28.8	82.8 ± 41.7	-9.1	2.20E-09
*Se*CHRIV	73671	74813	YDL211C	YDL211C	Protein of unknown function	36.0 ± 2.8	3.4 ± 0.2	-10.7	1.21E-53
*Se*CHRXI	493149	493970	*DAL80*	*YKR034W*	Negative regulator of multiple nitrogen catabolic genes	25.8 ± 4.4	2.1 ± 0.6	-12.3	8.69E-34
*Se*CHRII	925500	930622	*HXT3*	*YDR345C*	Low-affinity glucose transporter	541.0 ± 73.3	34.7 ± 1.8	-15.6	1.55E-163
*Se*CHRVII	692475	693617	*CLB6*	*YGR109C*	B-type cyclin	21.3 ± 3.7	1.2 ± 0.7	-17.6	7.11E-37
*Se*CHRVII	892340	893290	*RTA1*	*YGR213C*	Involved in 7-aminocholesterol resistance	82.9 ± 1.1	2.5 ± 0.8	-33.2	2.70E-106

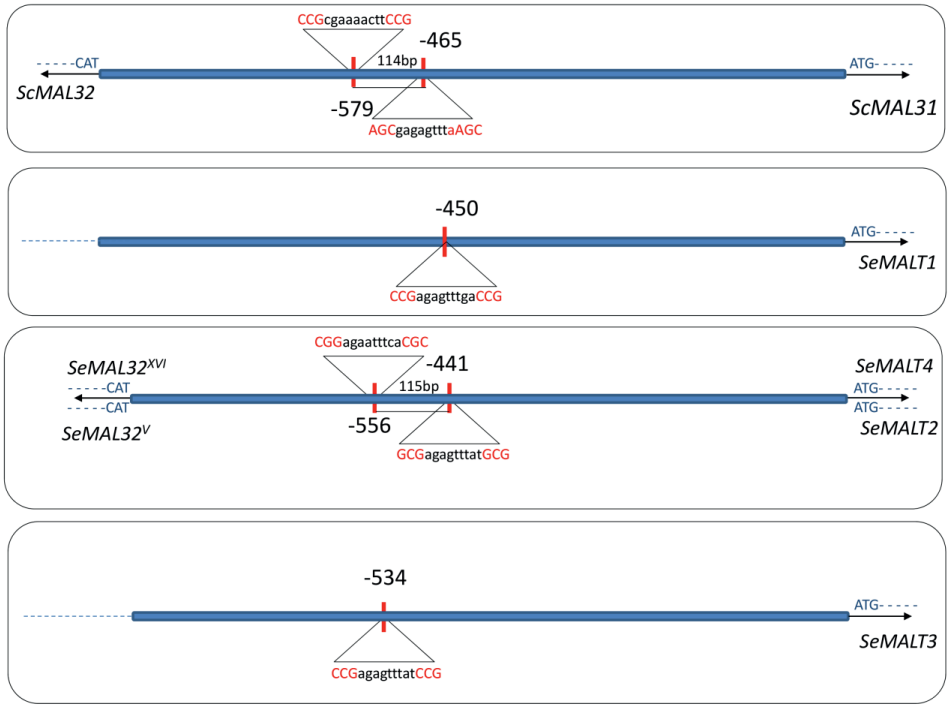

Figure S8: Comparison of divergent promoter elements located near maltose transporter genes at *MAL* loci of *S. cerevisiae* and *S. eubayanus* CBS 12357^{T}.

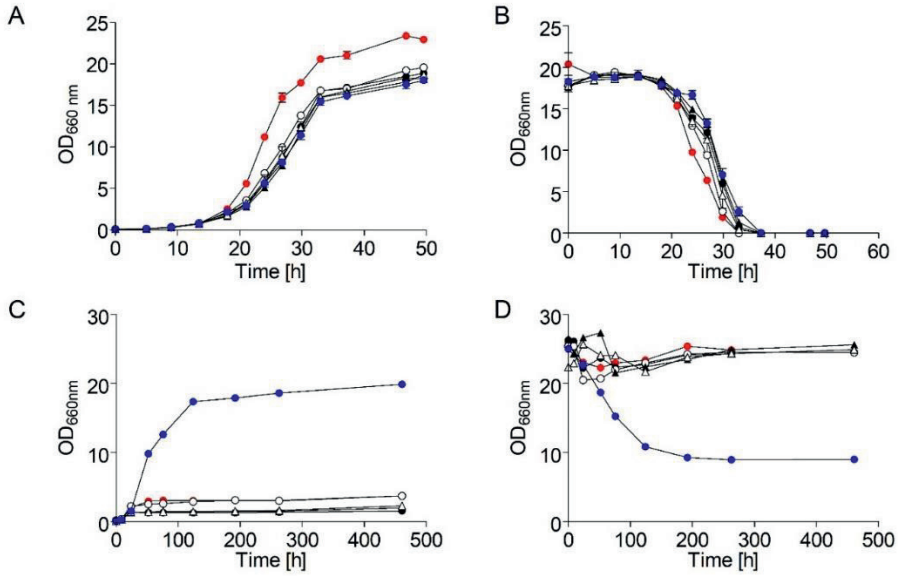

Figure S9: Characterization of the *S. cerevisiae* strains (●) IMZ616 [200], (○) IMX1253 (Sc*TEF1*$_{pr}$-*SeMALT1*-*ScCYC1*$_{ter}$), (▲) IMX1254 (Sc*TEF1*$_{pr}$-*SeMALT2*-*ScCYC1*$_{ter}$), (△) IMX1255 (Sc*TEF1*$_{pr}$-*SeMALT3*-*ScCYC1*$_{ter}$), (●) IMX1365 (Sc*TEF1*$_{pr}$-*ScMAL11*-*ScCYC1*$_{ter}$) and (●) *S. eubayanus* CBS 12357^{T} for growth on maltotriose. The strains were grown in SM$_{U}$G and SM$_{U}$M at 20 °C. Growth on glucose **A** and on maltotriose **C** was monitored based on optical density measurements at 660 nm (OD_{660nm}). Concentrations of glucose **B** and maltotriose **D** in culture supernatants were measured by HPLC. Data are presented as average and standard deviation of three biological replicates.

Chapter 3: *S. cerevisiae* x *S. eubayanus* interspecific hybrid, the best of both worlds and beyond

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Essentially as published in **FEMS Yeast Research** 2015, doi: 10.1093/femsyr/fov005

Abstract

Saccharomyces pastorianus lager-brewing yeasts have descended from natural hybrids of *S. cerevisiae* and *S. eubayanus.* Their alloploidy has undoubtedly contributed to successful domestication and industrial exploitation. To understand the early events that have led to the predominance of *S. pastorianus* as lager-brewing yeast, an interspecific hybrid between *S. cerevisiae* and *S. eubayanus* was experimentally constructed. Alloploidy substantially improved the performance of the *S. cerevisiae* × *S. eubayanus* hybrid as compared to either parent regarding two cardinal features of brewing yeasts: tolerance to low temperature and oligosaccharide utilization. The hybrid's *S. eubayanus* subgenome conferred better growth rates and biomass yields at low temperature, both on glucose and on maltose. Conversely, the ability of the hybrid to consume maltotriose, which was absent in the *S. eubayanus* CBS12357 type strain, was inherited from its *S. cerevisiae* parent. The *S. cerevisiae* × *S. eubayanus* hybrid even outperformed its parents, a phenomenon known as transgression, suggesting that fast growth at low temperature and oligosaccharide utilization may have been key selective advantages of the natural hybrids in brewing environments. To enable sequence comparisons of the parental and hybrid strains, the genome of *S. eubayanus* CBS12357 type strain (Patagonian isolate) was resequenced, resulting in an improved publicly available sequence assembly.

Background

Its relatively simple life cycle and ease of manipulation, combined with the frequent occurrence of natural hybrids, have made the genus *Saccharomyces* a paradigm for evolution of eukaryotic genomes [166]. Although the seven species of the *Saccharomyces* genus (i.e. *S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola*, *S. uvarum* and the newly identified *S. eubayanus*, [74]) show significant divergence at the nucleotide-sequence level, they show hardly any prezygotic barriers and, upon mating, form viable diploids [166]. This compatibility for sexual reproduction explains the rich, reticulate evolution of the *Saccharomyces* genus, and more particularly that of industrially relevant species. During reticulate evolution, prolonged local adaptation of the parents is unlikely to lead to hybrid offspring that have a competitive advantage in stable environments. However, domestication may have generated new niches that do offer specific advantages to hybrids [244, 245].

It is now well established that many natural and commercial wine and cider yeasts are double *S. cerevisiae* × *S. bayanus* or *S. cerevisiae* × *S. kudriavzevii* hybrids or even triple *S. cerevisiae* × *S. uvarum* × *S. kudriavzevii* hybrids [166, 246-248]. One of the best-known and most commercially relevant interspecific hybrids, the lager-brewing yeast *S. pastorianus*, is an alloploid hybrid of *S. cerevisiae* and *S. eubayanus* [81]. *Saccharomyces pastorianus* is a heterogeneous species, whose alloploidy has undoubtedly contributed to its industrial exploitation. Two major groups of lager-brewing yeast, the Saaz and Frohberg lineages (also called hybrid Group 1 and hybrid Group 2, respectively), have been identified and are proposed to be derived from two distinct hybridization events between *S. cerevisiae* ale strains and *S. eubayanus* [74, 76]. However recent identification of identical break point reuses (cross-overs resulting in *S. cerevisiae*–*S. eubayanus* chimeric chromosomes) in two genes (*HSP82* and *KEM1*) in both Group 1 and 2 strains would suggest that both lineages originate from a single hybridization and therefore would share a common hybrid ancestor [77]. These hybridization events occurred centuries ago and the selection pressure resulting from the brewing environment has triggered extensive genome reorganization, resulting in partial loss of heterozygozity and chromosomal rearrangements [82, 83]. Lager-yeast genomes are therefore complex, and many aspects of their evolutionary history remain to be elucidated.

Several 'artificial' hybrids have been experimentally constructed between *S. cerevisiae* and various of its *Saccharomyces* relatives, either to investigate fundamental aspects of speciation and reproductive isolation (*S. cerevisiae* × *S. paradoxus* hybrid, [249]) or for applied perspectives by creating hybrids with novel and potentially industrially relevant properties [250, 251]. More recently, using *S. cerevisiae* × *S. uvarum* de novo constructed hybrids, [252] demonstrated the value of these interspecific hybrids to unravel the context dependency of genome dynamic changes and to bring insight on

the evolutionary forces that have shaped the genome of the modern *Saccharomyces* hybrids. Heterosis (hybrid vigor) of the hybrid offspring in brewing environment has been ascribed to a beneficial combination between the cold tolerance of *S. eubayanus* and the strong fermentative metabolism of sugars of *S. cerevisiae* [81]. The veracity of this hypothesis can be relatively simply investigated by the construction of *S. cerevisiae* × *S. eubayanus* hybrids. When performing this study, no artificial *S. cerevisiae* × *S. eubayanus* hybrids had been reported in the literature. Since then, *S. cerevisiae* x *S. eubayanus* hybrids have been constructed and studied with regard to the genetics of lager beer brewing, the evolutionary history and industrial application of those hybrids (compare Chapter 1, p 27, 28; ref. 34, 113 - 118).

In the present study, mass mating was used to construct an hybrid between a haploid *S. cerevisiae* strain of the CEN.PK family [201] and a haploid strain derived from the *S. eubayanus* type strain CBS12357 (isolated from fruiting bodies of *Cyttaria hariotii* growing on Nothofagus trees in Patagonia [74]). To enable a detailed analysis of the *S. cerevisiae* × *S. eubayanus* hybrid's genome, its *S. eubayanus* parent CBS12357 was resequenced to obtain a high-quality, well-annotated and publicly available genome sequence. The performance of the de novo constructed hybrid was compared to that of its parents in controlled bioreactor cultures on defined media. Lager-beer production with *S. pastorianus* is performed under anaerobiosis at low temperatures (10–15°C) and with complex media (i.e. wort) containing mixtures of mono-, di- and trisaccharides [37, 253]. Specific growth rates and sugar consumption rates of the hybrid and its parents were therefore investigated over a wide temperature range (8–35°C) under strict anaerobiosis. Furthermore, the ability of the hybrid and its parents to anaerobically consume maltose and maltotriose was evaluated.

Materials and methods

Strains and maintenance

The yeast strains used in this study are shown in Table 1. Stock cultures were made by growing strains in 500-mL shake flasks, containing 100 mL YPD medium at 30°C and shaken at 200 rpm. Biomass concentration was evaluated by OD_{660} measurement, and fully-grown cultures were supplemented with 30% (v/v) glycerol, divided in 1 mL aliquots and stored at −80°C until further use.

Media

YPD medium contained 10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone and 20 g L^{-1} glucose. Synthetic medium (SM) contained 3.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 1 mL L^{-1} trace element solution and 1 mL L^{-1} vitamin solution (as

described in [254]). The pH of SM was set to 6.0 using 2 M KOH. When glucose was used as sole carbon source, 20 g L^{-1} of glucose was added to plates and shake flasks and 25 g L^{-1} to bioreactors (SM-Glu). To investigate utilization of wort sugars in shake flasks and bioreactors, a sugar mixture (dried glucose syrup C* plus 01987, Cargill, Haubourdin, France; sugar content (w/w): glucose 2.5%, maltose 28.0%, maltotriose 42.0%, higher saccharides 26.4%) was added to SM to a final total concentration of fermentable sugars of 20 g L^{-1} (SM-Mix). For cultivation on plates, media was supplemented with 2% agar. To trigger sporulation, diploid yeast strains were incubated in sporulation medium (2% potassium acetate, pH 7.0; [255]). To select for the presence of the *KanMX* gene, G418 was added to SM-Glu plates at a final concentration of 200 mg L^{-1}. To prevent inactivation of G418 by acidification of the medium, ammonium sulfate was replaced by 2.3 g L^{-1} urea and 6.6 g L^{-1} K_2SO_4 in G418-containing media. For bioreactor cultures, SM was supplemented with the anaerobic growth factors ergosterol and Tween 80 (0.01 g L^{-1} and 0.42 g L^{-1}, respectively; [254]) and with 0.15 g L^{-1} of antifoam C (Sigma-Aldrich, Zwijndrecht, the Netherlands).

Table 1 *Saccharomyces* strains used in this study.

Strain	Species	Relevant genotype	Ploidy	Reference
CEN.PK113-7D	*S. cerevisiae*	*MATa URA3*	Haploid	[93, 201]
CEN.PK122	*S. cerevisiae*	*MATa/MATα URA3/URA3*	Diploid	[201]
CR85	*S. kudriavzevii*	Unknown	Diploid	[296]
IMK439	*S. cerevisiae*	*MATα ura3Δ::KanMX*	Haploid	[297]
CBS12357	*S. eubayanus*	Unknown	Diploid	[74], CBS*
CBS1483	*S. pastorianus*	Unknown	Aneuploid	CBS*
IMS0408	*S. cerevisiae x S. eubayanus*	*MATa/MATα SeubURA3/Scura3Δ*::KanMX	Diploid	This study

*www.cbs.knaw.nl

Construction of interspecific hybrid

Stationary phase cells of *S. eubayanus* CBS12357 grown on YPD at 30°C were collected, washed and incubated for 64 h at 30°C in sporulation medium. Presence of asci was determined microscopically. A total of 100 µL of this culture was treated with zymolyase (5 U·mL^{-1}) for 15 min, after which spores were washed with sterile demineralized water and resuspended in 20 mL YPD medium. To this spore suspension, 100 µL of a mid-exponential culture of *S. cerevisiae* IMK439 (*ura3::KanMX*), grown in YPD at 30°C, was added. After incubation for 4 h at 30°C, interspecific hybrids were selected by streaking on a plate containing SM-Glu without uracil and with G418. This medium should only enable growth if both parental genomes are present. Single colonies were

selected and restreaked three times on selective plates containing SM-Glu without uracil and with G418, after which one colony was picked to inoculate a YPD shake flask. This interspecific hybrid strain was stored as described above and named IMS0408. Confirmation of its hybrid nature was performed after three repeated batch cultures at 37°C (a non-permissive temperature for *S. eubayanus*, see 'results' section) by flow cytometry, whole-genome sequencing, chromosome separation using contour-clamped homogeneous electric field (CHEF) electrophoresis and PCR.

DNA content determination by flow cytometry

Samples of culture broth (equivalent to circa 107 cells) were taken from mid-exponential shake-flask cultures on YPD and centrifuged (5 min, 4700 × g). The pellet was washed once with cold phosphate buffer (NaH_2PO_4 3.3 mM, Na_2HPO_4 6.7 mM, NaCl 130 mM, EDTA 0.2 mM; [256]), vortexed briefly, centrifuged again (5 min, 4700 × g) and suspended in 800 µL 70% ethanol while vortexing. After addition of another 800 µL 70% ethanol, fixed cells were stored at 4°C until further staining and analysis. Staining of cells with SYTOX® Green Nucleic Acid Stain (Invitrogen S7020) was performed as described [257]. Samples were analyzed on a Cell Lab Quanta™ SC MPL flow cytometer equipped with a 488 nm laser (Beckman Coulter, Woerden, the Netherlands). The fluorescence intensity (DNA content) was represented using the free CyFlogic software (version 1.2.1, ©Perttu Terhu & ©CyFlo Ltd).

Chromosome separation using CHEF electrophoresis

Agarose plugs containing the DNA of different strains were prepared using the CHEF yeast genomic DNA plugs Kit (Bio-Rad, Richmond, CA, USA) following manufacturer's recommendations and used for CHEF electrophoresis. The plugs were placed in a 1% megabase agarose gel in TBE buffer (5.4 g trizma base, 2.75 g boric acid, 2 mL of 0.5 M EDTA pH 8.0 and 1 L demineralized water) gel. For chromosome separation, the CHEF-DRIII pulsed field electrophoresis system (Bio-Rad, Richmond, USA) was used following the manufacturer's recommendations. For size markers, CHEF DNA Size Marker #170-3605 (Bio-Rad) was used.

Polymerase chain reaction (PCR)

Genomic DNA was extracted using the YeaStar™ Genomic DNA kit (Zymo Research Corporation, Irvine, CA, USA) following the manufacturer's recommendations. DNA concentrations were measured on a NanoDrop 2000 spectrophotometer (wavelength 260 nm) (Thermo Scientific, Wilmington, DE, USA). Multiplex PCR was performed with DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific). Primers

specific for *S. cerevisiae* (Scer F2: GCGCTTTACATTCAGATCCCG AG and Scer R2: TAAGTTGGTTGTCAGCAAGATTG) and *S. eubayanus* (Seub F3: GTCCCTGTACCAATTTAATATTGCGC and Seub R2: TTTCACATCTCTTAGTCTTTTCCAGACG), as described by [258], were used at a concentration of 300 nM. Cycling parameters were 94°C for 2 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, followed by a 10 min incubation at 72°C. A total of 10 µL samples from each reaction were analyzed by electrophoresis on a 2% (w/v) agarose gel in 0.5× TBE buffer (45 mM Tris-borate pH 8.0 1 mM EDTA), supplemented with SERVA DNA stain G (SERVA Electrophoresis, Heidelberg, Germany) for 40 min at 120 V.

Shake-flask experiments

Shake-flask cultures were grown in 500 mL flasks with a working volume of 100 mL. Temperature and shaking (200 rpm) were controlled in an Innova® 44 incubator shaker (Eppendorf, Nijmegen, the Netherlands).

Anaerobic batches and sequential batch reactors (SBR)

Batch cultivations were performed in 2-L laboratory bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1 L. The pH was controlled at 5.0 by the automated addition of 2 M KOH, and the stirrer speed was kept at 800 rpm. To ensure anaerobic conditions, bioreactors and medium vessels were continuously sparged with pure nitrogen (N_2) gas at flow rates of 0.7 L min^{-1} and ca. 7 mL min^{-1}, respectively, and equipped with Norprene tubing (Saint-Gobain Performance Plastics, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, the Netherlands). Active temperature regulation was performed and measured online by connecting a platinum resistance thermometer, placed in a socket in the bioreactor, to an RE304 low-temperature thermostat (Lauda, Lauda-Königshofen, Germany). To evaluate utilization of sugar mixtures by different yeast strains, batch cultivations were performed using SM-Mix at 20°C. Inocula were prepared by growing the individual strains at 20°C in shake flasks containing SM-Mix. Each batch experiment was performed in two independent culture replicates.

Anaerobic SBR cultivation was performed as previously described [259]. To control the transition between successive batches, carbon dioxide levels were used to automatically control the emptying and refilling of the reactors. CO_2 concentration in the exhaust gas below 0.05% indicated depletion of glucose and triggered the removal of 0.9 L of culture, leaving ca. 0.1 L of fermentation broth as inoculum for the subsequent batch. To start a new batch, the reactor volume was restored to 1 L by automated addition of fresh anaerobic medium. Growth was constantly monitored via online CO_2 measurements

in the off-gas. To evaluate the temperature range for optimal growth, yeast strains were cultivated in anaerobic SBRs at various temperatures on SM-Glu. Reactors were inoculated with precultures grown overnight under aerobic environment in shake flasks containing SM-Glu at 30°C for *S. cerevisiae* CEN.PK122, *S. pastorianus* CBS1483 and *S. cerevisiae* × *S. eubayanus* IMS0408 and at 24°C for *S. eubayanus* CBS12357. Physiology of the yeast strains was evaluated at temperatures ranging from 8 to 35°C. A whole temperature range was performed during each SBR, and each temperature cycle was maintained for two or three successive batches. To avoid evolution of strains resulting from a continuous selection pressure for high- or low-temperature tolerance, the sequence of the temperatures was chosen in such a way that the temperature neither increased nor decreased for more than three cycles in a row. To check whether the sequence in which the temperatures were analyzed affected the experimental outcome, the first and last cycles were performed at the same temperature. Comparison of the growth phenotype during these two temperature cycles revealed no significant differences in growth rate. The maximum specific growth rate (μ_{max}) was always calculated from the second batch at each temperature and based on continuous CO_2 measurements in the off-gas. To investigate the impact of low temperature on *S. cerevisiae* CEN.PK122, *S. eubayanus* CBS12357, the interspecific hybrid IMS0408 and *S. pastorianus*, a complete quantitative physiological characterization at 12 and 30°C was performed during the second batch of SBRs.

Analytical methods

Gas analysis, microscopy, analyses of biomass dry weight and extracellular metabolite concentrations were analyzed as described previously [259]. CO_2 in the off-gas was continuously monitored with an NGA 2000 analyzer (Rosemount Analytical, Orrville, OH, USA).

Calculations and statistical analysis of physiological characteristics

Maximum specific growth rates (μ_{max}), biomass yield on substrate ($Y_{X/S}$ in gDW·mmol glucose^{-1}), product yields on substrate ($Y_{i/S}$ in mmol·mmol glucose^{-1}) and biomass-specific product formation yields ($Y_{i/X}$ in mmol product·gDW^{-1}) were calculated via linear regression on at least five experimental data points. Maximum biomass-specific glucose consumption rates (q_S^{max}) were calculated by dividing μ_{max} by $Y_{X/S}$ and maximum biomass-specific production rates were calculated by multiplying $Y_{i/X}$ by μ_{max}, based on the assumption that growth stoichiometries remained constant in exponential-phase cultures. CO_2 yields on glucose could not be reliably estimated with SBRs due to small variations in the starting volume of each batch.

The standard error of the mean was calculated following equation (1):

$$SE_{\bar{Y}iX} = \sqrt{\frac{s^2_{Y_{iX,1}} + s^2_{\bar{Y}_{iX,2}}}{4}}$$

(1)

in which the standard deviation ($s_{YiX,j}$) was calculated by use of the Microsoft Excel™ linest function (linear regression) on at least five experimental data points. Statistical significance was evaluated using an independent two-sample t-test, assuming unequal variance and equal or unequal sample size (also known as Welch's t-test) according to equation (2).

$$t = \frac{\bar{Y}iX_A - \bar{Y}iX_B}{S_{\bar{Y}iX,AB}}$$

(2)

in which $S_{\bar{Y}iX,AB} = \sqrt{(SE_{\bar{Y}iX_A})^2 + (SE_{\bar{Y}iX_B})^2}$.

The degrees of freedom used for significance testing was calculated according to the Welch–Satterthwaite equation (equation 3; [260]):

$$df \approx \frac{\left(\frac{s_{\bar{Y}iX_A}{}^2}{n_A} + \frac{s_{\bar{Y}iX_A}{}^2}{n_B}\right)^2}{\frac{s_{\bar{Y}iX_A}{}^4}{n_A \cdot df_A} + \frac{s_{\bar{Y}iX_B}{}^4}{n_B \cdot df_B}}$$

(3)

with n being the number of replicas in Y_{iX} (inour case 2) and df_A and df_B the degrees of freedom in respectively condition A and B. Differences with a *P*-value < 0.05 were considered significant. Equations (1–3) are specific for the average biomass specific substrate consumption and product formation yields (Y_{iX}); however, the same methodology was applied for comparison of the yields on substrate (Y_{Si}).

Whole-genome sequencing and analysis of the *S. eubayanus* CBS12357 and of the interspecific hybrid IMS0408

Genome sequencing was performed using Illumina HiSeq2000 (Illumina, San Diego, CA, USA) at Baseclear (Leiden, the Netherlands). Genomic DNA of *S. eubayanus* CBS12357 strain was isolated using a YeaStar Genomic DNA kit (Zymo Research, Irvine, CA, USA) and used to obtain a 50-cycle mate pair library with an 8-kb insert size. For a second library, fragments of ~180–200 bp were sequenced paired-end with a read length of 100 bp. Overlapping read pairs were merged into single longer (pseudo) reads, yielding for both libraries over 2200 Mb total sequence, corresponding to a ca. 185× coverage. Genome assembly was performed on the (pseudo)reads library using the GSAssembler 2.6, also known as Newbler (454 Life Sciences/Roche), using default settings. Mate pair

scaffolding was performed on the assembled contigs using SSPACE (version 2.0, [261]) with Bowtie (version 0.12.5, [262]) as mapping tool. Based on the paired link information between the different contigs, the orientation and distance between consecutive contigs were determined and the contigs were merged into scaffolds. Gaps were introduced during scaffolding and represented by the character N for every base to preserve the distance between two placed contigs. Gapfiller [263] was applied and closed 119 out of 286 gaps, solving 17252 unknown bases. The raw read sequences and the assembled contigs have been deposited under the BioProject PRJNA264003 at NCBI (http://www.ncbi.nlm.nih.gov/bioproject).

The ratio of heterozygosity was calculated as follows: the sequencing reads were first remapped to the assembled scaffolds using SAMtools [264]. The binary alignment matrix file (.bam) was subsequently processed using GATK [265]. The list of heterozygous positions was filtered to exclude positions with sequencing coverage lower than 20-fold and with variation ratio below 0.2. The heterozygosity ratio was calculated as the density of heterozygous positions over the haploid genome size (11.9 Mb).

The genomic DNA of the hybrid IMS0408 strain was used to obtain a 100-cycle paired-end library with an 280-bp insert size using Illumina HiSeq2000 (Illumina, San Diego, CA, USA). The library yielded a total of 5.7 Mb pair sequence, corresponding to ca. 20× coverage. The reads of the hybrid strain IMS0408 were mapped on to the reference genome sequences of *S. cerevisiae* CEN.PK113-7D (accession number: PRJNA52955) and *S. eubayanus* CBS12357 using Burrows–Wheeler Aligner algorithm with default settings [216, 266]. The raw sequence data of the *S. cerevisiae* × *S. eubayanus* hybrid IMS0408 have been deposited under the BioProject PRJNA264003 at NCBI. The Magnolya algorithm was used to analyze copy number variation, using Newbler (454 Life Sciences) for assembly [267].

The sequences of *S. pastorianus* WS34/70 (PRJNA29791, [83]) and of *S. eubayanus* CDFM21L.1 (PRJNA254367, [108]) were used for the comparative analysis of the MAL genes.

Results

Resequencing of *S. eubayanus* CBS12357

Assembly of the available sequence data of *S. eubayanus* [74] resulted in a highly fragmented genome. To enable and facilitate analysis of the genome sequence of constructed *S. cerevisiae* × *S. eubayanus* hybrids, an improved sequence assembly of *S. eubayanus* was necessary. Two different libraries were prepared and sequenced using Illumina technology. The first sequence data set was a 100-cycle paired-end library with an expected insert size of 180 bp. The overlapping read pairs data were merged into 3 293 656 single longer 'pseudo' reads of 143 ± 20.5 bp, which represented a total

sequence length of 471 Mb. These were subsequently assembled into 372 contigs with a size of 500 bp or longer, resulting in a total sequence of 11.49 Mb (Table 2). In a second assembly step, the 50-cycle mate pair library (17 521 927 pairs) with an 8-kb insert size, representing 1.75 Gb of sequence information, was used to further structure the *S. eubayanus* CBS12357 strain genome sequence. Scaffolding enabled a reduction of the number of contigs from 372 to 76 (Table 2), which led to a slightly larger haploid genome size of 11.9 Mb, sequenced with a coverage depth of 185-fold. The obtained scaffolded genome sequence was annotated using the MAKER2 pipeline [217] resulting in the annotation of 5238 ORFs.

Table 2 Genome sequencing statistics for libraries, assembly, scaffolding and gap filling.

Assembly	
Number of contigs (≥ 500bp)	372
Avg contig size (kbp)	30.9
N50 (kbp)	94.1
Largest contig (kbp)	463.9
Total sequence (Mbp)	11.5

Scaffolding and Gapfilling	
Number of scaffolds (≥ 500bp)	76
Avg scaffold size (kbp)	156.7
N50 (kbp)	730.2
Largest scaffold (Gbp)	1.3
Genome size (Mbp)	11.9*

*of which 393002 N's in 167 gaps

As the *S. eubayanus* CBS12357 strain sequenced in this study should be identical to the strain sequenced by Libkind and co-workers, we verified that the original sequencing data (Short Read Archive SRP006155 of SRA030851) could be mapped on the resequenced assembly. 98% of the original reads perfectly mapped (no mismatch, no gap allowed) onto the resequenced genome, thereby confirming strain identity. Relative to *S. cerevisiae*, *S. eubayanus* harbors reciprocal translocations between chromosomes VIII and XV, and between chromosomes II and IV, probably due to ectopic recombinations between duplicated RPL2A and RPL2B genes and TY LTR elements, respectively [268]. Alignment of the *S. eubayanus* scaffolds on the *S. cerevisiae* chromosome template confirmed these occurrences in the *S. eubayanus* CBS12357 genome (Fig. 1A).

S. eubayanus CBS12357 is polyploid, as shown by its ability to sporulate (data not shown and [74]). Flow cytometry showed a DNA pattern compatible with diploidy (Fig. 1B) but was not sensitive enough to rule out aneuploidy for one or a few chromosomes. However, copy number estimation of contigs assembled from sequencing data [267] revealed a constant ploidy for all chromosomes (Fig. 1C). Taken together, these data demonstrate that *S. eubayanus* CBS12357 is a strict diploid.

The genome assembly of *S. eubayanus* CBS12357 resulted logically in the reconstruction of a haploid genome which however gives only a partial representation of

its diploid nature. To get a better overview of the difference between the gene alleles, the heterozygosity ratio was estimated. The analysis revealed 246 heterozygous positions exclusively found in upstream and downstream regions of 121 genes (Table S1, Supporting Information). Therefore, this corresponds to a very low heterozygosity ratio of 0.0021%, value very similar to the ratio of 0.0039% reported for the *S. eubayanus* strain CDFM21L.1 [108].

Construction of a *S. cerevisiae* × *S. eubayanus* hybrid, IMS0408

Interspecific hybrids between *S. cerevisiae* and *S. eubayanus* were constructed from haploid parents. While *Saccharomyces* strains of the CEN.PK family are heterothallic, it is yet unknown whether *S. eubayanus* CBS12357 is homo- or heterothallic and therefore whether pure lines of haploid mating types a and α can be obtained. To simplify and accelerate the construction of hybrids, a 'mass mating' strategy [269-271] between *S. cerevisiae* haploid vegetative cells and *S. eubayanus* spores was undertaken (Fig. 2A). True hybrids were selected based on their uracil prototrophy and G418 resistance, contributed by the *S. eubayanus* and *S. cerevisiae* parents, respectively (Fig. 2A). While hybridization can lead to genetic and phenotypic variation between offspring from identical parents, the diploid *S. eubayanus* parent, as shown in the previous section, has a very low heterozygosity ratio, and phenotypic variations between the

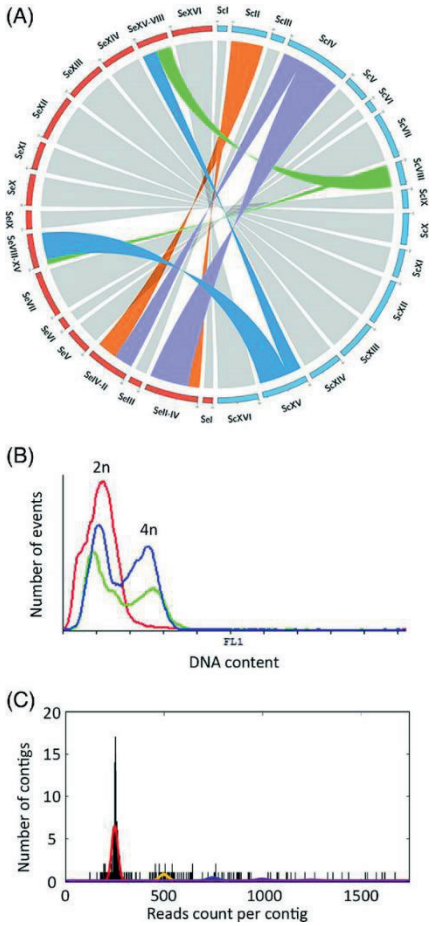


Figure 1. Chromosomal architecture of *S. eubayanus* CBS12357. (**A**) Circos plot representation of the sequencing data reveal that, when compared to *S. cerevisiae* (blue), *S. eubayanus* (red) harbours reciprocal translocations between chromosomes VIII and XV, and II and IV. (**B**) DNA content measured by flow cytometic analysis of haploid S. cerevisiae IMK439 (red); diploid *S. cerevisiae* CEN.PK122 (green) and *S. eubayanus* CBS12357 (blue). (**C**) de novo copy number detection using Magnolya [267]. The black bars represent the distribution of read counts per assembled contigs. Read counts have been normalized for visualization purposes to a contig length of 1000 bp. Copy numbers (CN) of the contigs were estimated using the poisson mixture model (PMM) algorithm in Magnolya. The PMM was fitted on the contigs and plotted as colored lines. The first peak (red line) represents 98% of the assembly showing a constant ploidy. The remaining 2% encompasses redundant sequences (transposons, paralogous genes) that were artificially merged by the assembler into single contigs, resulting in small peaks with large CN (yellow, blue lines).

constructed *S. cerevisiae* × *S. eubayanus* hybrids are thus not expected to be substantial. Therefore, a single interspecific hybrid between *S. cerevisiae* and *S. eubayanus*, named IMS0408, was chosen for further analysis. To ensure that the population obtained was solely composed of the hybrid and was not a mixed population of *S. cerevisiae* and *S. eubayanus* haploid strains, IMS0408 was grown in three sequential batches at 37°C which, as will be described below, is a non-permissive temperature for *S. eubayanus*. Culture purity was verified by multiplex PCR (Fig. 2B) and flow cytometry analysis of the DNA content of IMS0408 was consistent with diploidy (Fig. 2C). Finally, karyotype analysis by CHEF showed that all chromosomes of the parental strains were present in the hybrid IMS0408 (Fig. 2D).

The genome of the hybrid strain IMS0408 was sequenced using Illumina technology as described above for S. eubayanus. A 280-bp insert size library was then sequenced to generate a 100-cycle paired-end dataset, representing a total sequence amount of 600 Mb. The paired-end data were mapped on the reference genomes of the parental strains S. cerevisiae CEN.PK113-7D and S. eubayanus CBS12357. Out of a total of 5 623 306 reads, 2 692 867 mapped to the CEN.PK113-7D sequence (47.9%) and 2 523 306 (45.6%) reads mapped to the CBS12357 sequence. These results further confirmed the hybrid nature of the genome of the isolate strain IMS0408. In good agreement with the DNA content, mapping of the sequence of IMS0408 to the reference genomes of the parental strains showed that both genomes were present in the same ratio, and therefore that the strain has an allodiploid genome of 24 Mb (Fig. 2E).

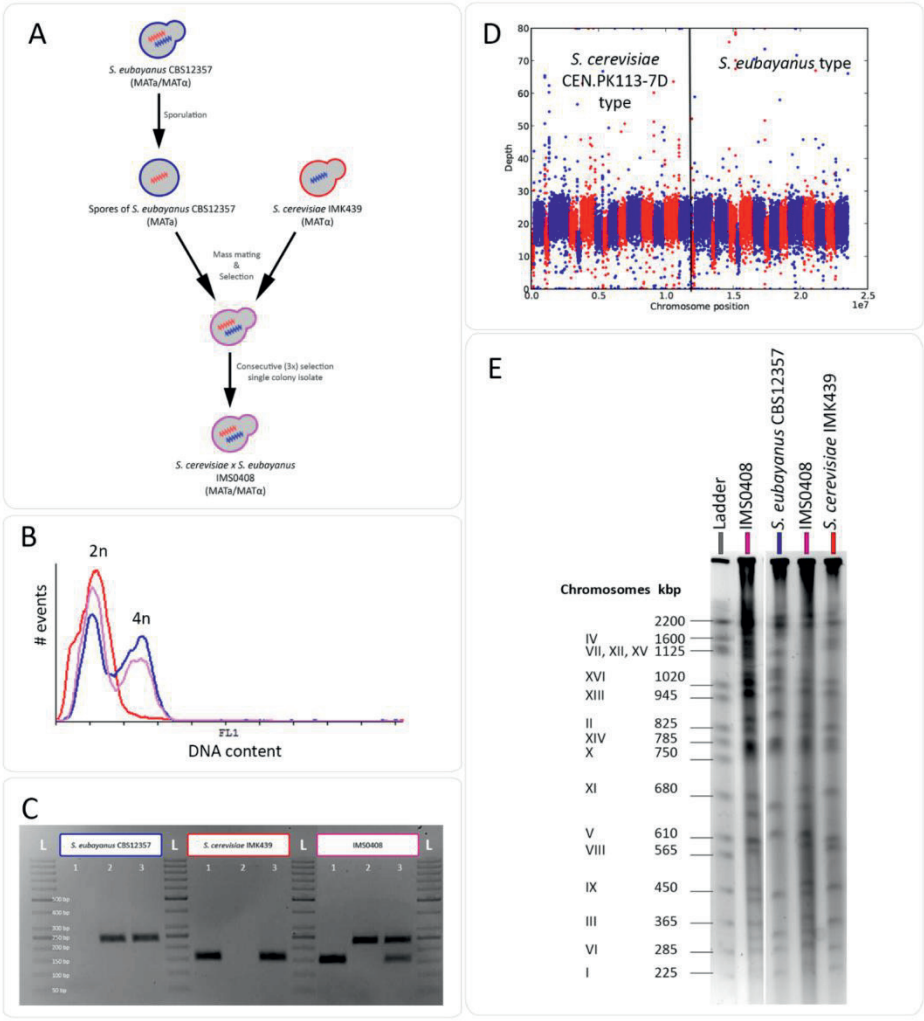


Figure 2. Construction and validation of the *S. cerevisiae* × *S. eubayanus* interspecific hybrid IMS0408. (A) Strategy for the construction of interspecific hybrids between *S. cerevisiae* IMK439 (*ura3*Δ::KanMX) and *S. eubayanus* CBS12357 (*SeubURA3*). **(B)** PCR confirmation of the presence of *S. eubayanus* and *S. cerevisiae* marker genes in IMS0408. Lane 1: *S. cerevisiae*'s specific primers. Lane 2: *S. eubayanus*' specific primers. Lane 3: multiplex PCR with primers specific for both parents. L: GeneRuler 50 bp DNA Ladder. **(C)** Ploidy assessment of the interspecific hybrid IMS0408. DNA content measured by flow cytometric analysis of *S. cerevisiae* IMK439, haploid (red); *S. eubayanus* CBS12357, diploid (blue) and the interspecific hybrid IMS0408 (pink). **(D)** Karyotyping of IMS0408 and of its parental strains. Chromosomes (Chr) numbers and sizes in kbp are shown and were obtained using *S. cerevisiae* YNN295 as ladder. **(E)** Mapping of the genome sequence of IMS0408 to the reference genomes of *S. cerevisiae* CEN.PK113-7D and *S. eubayanus* CBS12357.

Temperature tolerance of *S. cerevisiae*, *S. eubayanus* and their interspecific hybrid

To evaluate the temperature range for growth of the hybrid strain and to compare it to that of its parents, quantitative analysis of the physiology of the three strains was performed in anaerobic bioreactor batch cultures at temperatures ranging from 8 to 35°C. To enable acclimation to each temperature, SBRs were used [272, 273]. In SBRs, successive cycles of batch cultivation are performed by automatically emptying near-stationary phase cultures and refilling them with fresh medium. In each emptying step, a small volume of broth is retained in the bioreactor to serve as inoculum for the subsequent cycle.

The anaerobic SBR experiments demonstrated that *S. eubayanus* shares with *S. cerevisiae* and *S. pastorianus* the ability to grow in minimal chemically defined medium in the complete absence of oxygen [148, 274]. As expected, *S. cerevisiae* grew at all tested temperatures, with highest (0.41 ± 0.01 h^{-1}) and lowest (0.013 ± 0.003 h^{-1}) specific growth rates at 35°C and 8°C, respectively (Fig. 3). As previously described [74], *S. eubayanus* was more cryotolerant than *S. cerevisiae*, displaying a significantly (t-test P-value below 0.05) higher growth rate at temperatures below 25°C (Fig. 3). In contrast, *S. eubayanus* was unable to grow at 35°C and showed its highest specific growth rate of 0.28 ± 0.005 h^{-1} at a temperature of 30°C, which was 22% lower than the growth rate of *S. cerevisiae* at that temperature. Not only did the interspecific hybrid IMS0408 acquire the growth characteristics of the best performing parent for most temperatures, but it even outperformed the best parent for temperatures ranging from 20 to 30°C. IMS0408 was able to grow at 35°C, albeit with a 22% lower specific growth rate than *S. cerevisiae* (Fig. 3).

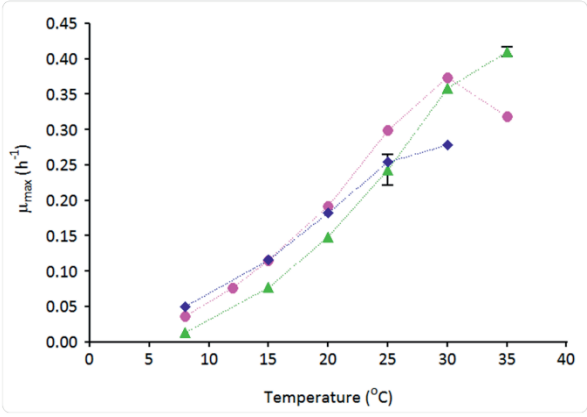


Figure 3. Temperature tolerance of the interspecific hybrid IMS0408 and its parents *S. cerevisiae* CEN.PK122 and *S. eubayanus* CBS12357. The maximum specific growth rate (μ_{max}) of *S. cerevisiae* CEN.PK122 (filled triangle), *S. eubayanus* CBS12357 (filed rhombus) and their interspecific hybrid IMS0408 (filled circle) at 8, 15, 20, 25, 30 and 35°C was determined in anaerobic SBRs using SM-Glu. μ_{max} was calculated during the second batch at each temperature from continuous CO_2 measurements in the off-gas. These data represent the average and standard error of the mean of independent duplicate cultures.

Quantitative physiology of *S. eubayanus*, *S. cerevisiae*, their interspecific hybrid and *S. pastorianus* grown at high and low temperatures

A quantitative physiological characterization was performed at low and high temperature in anaerobic SBRs. For these experiments, the high temperature was set at 30°C, as *S. eubayanus* cannot grow at 35°C (Fig. 3). The low temperature was set to 12°C, which is a relevant temperature for brewing (typical temperature range between 10 and 15°C) at which all strains still grew relatively well (around 0.05 h^{-1}).

Comparison of the parental strains revealed that despite the substantial lower specific growth rate of *S. eubayanus* as compared to *S. cerevisiae* at 30°C, the overall physiology of the two strains was very similar (Table 3, Table S2, Supporting Information). Indeed, while some differences were observed in the specific production rates and yields of minor fermentation products (i.e. pyruvate, lactate and acetate), the biomass, ethanol and glycerol yields were very similar for the two parents (less than 2% difference between strains). Conversely, cultivation at 12°C revealed marked differences. Not only did *S. eubayanus* grow twice as fast as *S. cerevisiae* at this temperature but it also displayed a 46% higher biomass yield on glucose. While the biomass yield on glucose of *S. cerevisiae* at 12°C was approximately 50% lower than at 30°C, the biomass yield of *S. eubayanus* was remarkably unaffected by low temperature. The different response to temperature of the two parental strains was also apparent in the production rates and yields of minor fermentation by-products (Table 3, Table S2, Supporting Information). In particular, the glycerol yield on glucose which, in *S. cerevisiae* was similar at 12°C than at 30°C (t-test P-value above 0.05), was temperature dependent in *S. eubayanus* and lower at 12°C than at 30°C (12% lower, t-test P-value below 0.05).

Correspondence of the physiology of the IMS0408 hybrid to each of its parents was temperature dependent. At 30°C, where the major difference between the parents resided in specific growth rate, and consequently in overall specific consumption and production rates, IMS0408 grew and metabolized glucose at rates similar to that of its *S. cerevisiae* parent (Table 3, Table S2, Supporting Information). Conversely, at 12°C, IMS0408 displayed growth characteristics (i.e. specific growth rate and biomass yield) that were similar to that of its *S. eubayanus* parent. Still, at 12°C, the *S. cerevisiae* ancestry was visible through the fermentation by-products, whose production yields were either close to those observed in *S. cerevisiae* cultures or intermediate between the two parents (Table 3).

To investigate how the growth characteristics of the artificial hybrid compared to that of a lager-brewing yeast, *S. pastorianus* CBS1483 was grown under the same conditions as the other three *Saccharomyces* strains. At 30°C, this *S. pastorianus* strain grew at a specific rate comparable to that of *S. eubayanus* and displayed biomass and

Table 3 Physiological parameters of *S. cerevisiae*, *S. eubayanus*, the *S.c.* x *S.e.* interspecific hybrid and *S. pastorianus* grown in SBR in SM-Glu at 12 and 30 °C. Average of two independent culture replicates and standard deviation of the mean are shown. CO_2 yields cannot be reliably measured with SBRs and are not presented (see Material and Methods section). DW, biomass dry weight.

	S. cerevisiae CEN.PK122		*S. eubayanus* CBS12357		*S. cerevisiae x S. eubayanus* IMS0408		*S. pastorianus CBS1483*	
	30°C	12 °C	30°C	12 °C	30°C	12 °C	30°C	12 °C
μ_{max} (h^{-1})	0.329±0.006	0.039±0,001	0.255±0.005	0.079±0.001	0.370±0.009	0.063±0.001	0.217±0.011	0.035±0.000
Biomass yield (g DW·mmol glucose^{-1})	0.0163±0.0003	0.0104±0,0003	0.0163±0.0002	0.0152±0.0001	0.0171±0.0004	0.0143±0.0002	0.0165±0.0009	0.0148±0.0002
Yields on glucose (mmol·mmol glucose^{-1})								
Ethanol yield	1.40±0.01	1.27±0,03	1.39±0.01	1.41±0.01	1.37±0.01	1.47±0.01	1.50±0.01	1.45±0.02
Glycerol yield	0.194±0.004	0.183±0,005	0.198±0.004	0.173±0.003	0.200±0.003	0.207±0.002	0.194±0.003	0.166±0.004
Pyruvate yield	0.0061±0.0001	0.0021±0,0001	0.0084±0.0001	0.0051±0.0001	0.00767±0.0001	0.0028±0.0000	0.0088±0.0000	0.0010±0.0001
Succinate yield	0.0077±0.0009	0.0029±0.0002	0.0068±0.0001	0.0058±0.0002	0.0083±0.0013	0.0048±0.0001	0.0049±0.0001	0.0102±0.0002
Lactate yield	0.0130±0.0004	0.0069±0.0002	0.0182±0.0004	0.0154±0.0002	0.0152±0.0001	0.0164±0.0002	0.0167±0.0003	0.0068±0.0001
Acetate yield	0.0235±0.0009	0.0515±0.0014	0.0170±0.001	0.0118±0.0005	0.0202±0.0013	0.0382±0.0003	0.0112±0.0011	ND[a]
Yields on biomass (mmol·g DW^{-1})								
Ethanol Yield	85.8±1.4	122.0±3.7	85.2±0.7	93.2±0.6	79.9±2.1	102.5±1.45	90.4±5.4	98.2±2.6
Glycerol Yield	11.9±0.2	17.6±0.5	12.2±0.3	11.4±0.2	11.6±0.1	14.5±0.23	11.7±0.7	11.2±0.4

product yields similar to that of both *S. cerevisiae* and *S. eubayanus*. Although *S. pastorianus* has been described as cryotolerant [80], at 12°C, this *S. pastorianus* strain grew significantly slower than *S. eubayanus* and at a rate that was similar to that of *S. cerevisiae* (Table 3). However, as observed for *S. eubayanus* and IMS0408, its biomass yield at 12°C was significantly higher than that of *S. cerevisiae* (Table 3). Regarding fermentation by-products, *S. pastorianus* displayed a slightly different pattern than the other strains (Table 3). As observed in *S. eubayanus*, the glycerol yield on glucose was temperature dependent and was substantially lower at 12°C than at 30°C.

The ability of the *S. cerevisiae* × *S. eubayanus* hybrid IMS0408 to consume maltotriose is inherited from its *S. cerevisiae* parent

The oligosaccharides maltose and maltotriose are the major carbon sources in wort [275]. *S. cerevisiae*, *S. eubayanus* and *S. pastorianus* can all utilize maltose as carbon source [74, 81, 276]. However, while *S. cerevisiae* and *S. pastorianus* are known to consume maltotriose, the ability of *S. eubayanus* to consume this trisaccharide is still a matter of debate [80, 134, 138, 145]. When grown at 20°C in anaerobic batch cultures on mixtures of glucose, maltose and maltotriose all three *Saccharomyces* species, as well as the interspecific hybrid, consumed maltose (Fig. 4). As expected, both *S. cerevisiae* and *S. pastorianus* consumed all three sugars. In contrast, *S. eubayanus* did not utilize the maltotriose present in the culture medium and its growth ceased upon depletion of glucose and maltose. The remarkable observation that glucose was not strictly preferred over maltose and maltotriose in these cultures is probably related to the precultivation of the inoculum, which was grown on the same sugar mixture. While *S. pastorianus* consumed all available maltotriose, *S. cerevisiae* left ca. 0.3 $g\,L^{-1}$ of maltotriose in the medium at the end of the experiment, suggesting a lower affinity for this sugar. *S. eubayanus* grew significantly faster (P-value below 0.05) on maltose than *S. cerevisiae* and *S. pastorianus* (μ_{max} of 0.118 ± 0.004 h^{-1}, 0.071 ± 0.000 h^{-1} and 0.085 ± 0.001 h^{-1}, respectively). It is noteworthy that the difference in growth rate between *S. cerevisiae* and *S. eubayanus* was substantially more pronounced during growth on maltose than during growth on glucose. Indeed, at 20°C, *S. eubayanus* grew ca. 20% and 66% faster on glucose and maltose than *S. cerevisiae*, respectively (Figs 3 and 4).

In the *S. cerevisiae* × *S. eubayanus* interspecific hybrid, the inability of *S. eubayanus* to consume maltotriose was compensated by the acquisition of the *S. cerevisiae* genome, as indicated by fast consumption of maltotriose by IMS0408 (Fig. 4). However, as observed for its *S. cerevisiae* parent, IMS0408 did not fully consume maltotriose but left a low concentration at the end of growth. From its *S. eubayanus* parent, the *S. cerevisiae* × S. *eubayanus* interspecific hybrid inherited the ability to grow more rapidly on maltose at 20°C. As observed for *S. eubayanus*, IMS0408 grew substantially faster than *S. cerevisiae* (μ_{max} of 0.124 ± 0.001 h^{-1} and 0.071 ± 0.000 h^{-1}

respectively), resulting in shorter fermentation times (approximately 10 hours shorter). Interestingly, the *S. cerevisiae* × *S. eubayanus* hybrid showed a pronounced diauxic utilization of maltose and maltotriose, reflected in the CO_2 profile (Fig. 4, two peaks), that was not observed in *S. cerevisiae*, which co-consumed these two sugars. This diauxic sugar utilization was also observed in *S. pastorianus*. The different final biomass concentrations in cultures of IMS0408 and *S. cerevisiae* grown on sugar mixtures (Fig. 4) were consistent with the higher biomass yield of the hybrid observed at low temperature on SM-Glu (Table 3).

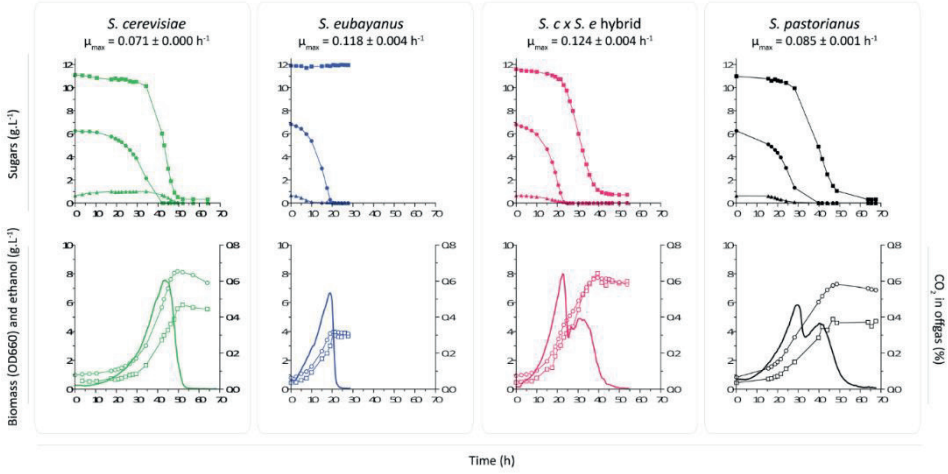


Figure 4. Oligosaccharides utilization by the *S. cerevisiae* × *S. eubayanus* hybrid IMS0408, *S. cerevisiae* CEN.PK122, *S. eubayanus* CBS12357 and the lager-brewing strain *S. pastorianus* CBS1483 in anaerobic batch at 20°C. The top graphs represent the concentration of maltotriose (squares), maltose (circles) and glucose (triangles). The bottom graphs represent the biomass density (squares), the% CO_2 in the off-gas (continuous line) and the ethanol concentration (circles). A single representative batch cultivation is shown for each strain. Duplicate experiments yielded the same results (Fig. S1, Supporting Information). Indicated specific growth rates (μ_{max}) were calculated from duplicate experiments based on OD measurements in early exponential phase while maltose was the major consumed carbon source. The average and standard error of the mean are reported.

Discussion

An improved genome sequence of *S. eubayanus* CBS12357

The original genome sequence data of *S. eubayanus* consist of a single library of 36-nucleotide reads. These reads have not previously been de novo assembled, but only mapped on reference genome sequences [74]. Assembly of this original data set using IDBA (Iterative De Bruijn graph de novo Assembler; http://i.cs.hku.hk/~alse/hkubrg/projects/idba/), an assembler compatible with very short reads, produced 14418 contigs, resulting in a highly fragmented genome. This

rendered the interpretation and the subsequent utilization extremely difficult. Since these sequence data were obtained, major improvements in next-generation sequencing methodologies have enabled increased sequence read length. Additionally, the combination of a paired-end library with short insert size, which enabled collapsing paired reads into longer continuous sequences, and a mate-paired library with long (8-kb) insert sizes enabled a new, de novo assembly of the *S. eubayanus* genome. This genome presents a vastly greater long-range continuity and fewer gaps than the previously available genome sequence (Table 1) allowing identification of key genes such as maltose transporter genes (discussed below). Despite clear improvements, the number of annotated genes is probably still significantly underestimated because the use of short-read sequences did not allow full assembly of most repeated sequences in the *S. eubayanus* genome. This notwithstanding, availability of the raw data and the structured, annotated sequence of *S. eubayanus* CBS12357 should benefit the yeast research community.

Physiological evidence of cold adaptation of *S. eubayanus*

Temperature has been implicated as a key environmental factor in the speciation of the *Saccharomyces* genus, with the different temperature optima of the seven *Saccharomyces* species enabling their co-existence in specific ecological niches [277-279]. *S. cerevisiae*, the most 'thermotolerant' species of the genus, has maximum and optimum growth temperatures of ca. 41°C and 33°C, respectively, in aerobic cultures [280]. The present study shows that this temperature range is similar in anaerobic cultures. The scarcely characterized species *S. eubayanus* was first isolated from Nothofagus trees and their surrounding environment [74]. The present study demonstrates that, similar to other *Saccharomyces* species [281], *S. eubayanus* is capable of anaerobic growth in chemically defined minimal medium. Moreover, its previously reported ability to grow faster than *S. cerevisiae* at low temperatures [74] was shown to be oxygen independent. Remarkably, at low temperature *S. eubayanus* grew even faster than *S. kudriavzevii* (Fig. S2, Supporting Information), previously considered to be the most cold tolerant *Saccharomyces* species [278, 279]. *Saccharomyces eubayanus* CBS12357 did not only grow faster than *S. cerevisiae* at temperatures below 25°C but it also exhibited a higher biomass yield on glucose at low temperature (Table 3). While this observation supports the notion that *S. eubayanus* has adapted to cold environments, the present study does not allow conclusions on the molecular basis for its higher biomass yield. Future studies on this characteristic should in particular focus on possible differences in biomass composition and, in particular, membrane composition of these two *Saccharomyces* species. Differences in membrane composition could result in different rates of ion diffusion across cellular membranes and thereby affect maintenance energy requirements [282]. In this respect, it is interesting to note that cold-adapted *S. kudriavzevii* was found to have a different lipid composition than *S. cerevisiae* [283,

284]. The production of glycerol, known cryoprotectant in *S. cerevisiae* [285-288], has previously been proposed to contribute to the cold tolerance of *S. kudriavzevii* [285-290]. However, *S. eubayanus* produced less glycerol at low temperature (Table 3), suggesting that glycerol production does not play an important role in this species. The lower glycerol production and higher biomass yield at low temperature of *S. eubayanus* as compared to *S. cerevisiae* were shared by *S. pastorianus*.

While the present data are not sufficient to identify the underlying mechanisms that govern the physiological differences between the *Saccharomyces* species at different temperatures, the improved *S. eubayanus* sequence and the availability of the *S. cerevisiae* × *S. eubayanus* hybrid should in the future contribute to deciphering the multifactorial and poorly understood molecular basis of cold tolerance.

On the origin of maltotriose assimilation in lager-brewing strains

MAL11/AGT1 has been proposed to encode the transporter responsible for maltotriose uptake in *S. cerevisiae* [143]. The identification in lager-brewing strains of a frame shift leading to an early stop codon and a non-functional *S. cerevisiae* allele of *MAL11/AGT1* have led to the conclusion that the ability of *S. pastorianus* to utilize maltotriose was not inherited from *S. cerevisiae* but from the *S. eubayanus* ancestor [134, 138, 145]. The results reported in the present study are consistent with a recent report [80] contradicting this in silico-based hypothesis. A close inspection of the de novo assembled and annotated *S. eubayanus* genome sequence identified three *MAL* loci, on scaffolds 4, 7 and 12. All three *MAL* genes exhibited higher similarity to *MAL31* than to *MAL11/AGT1* (Fig. 5), consistent with the absence of functional maltotriose transporters in *S. eubayanus* CBS12357. While our results suggest that the *S. cerevisiae* subgenome, and not *S. eubayanus*, conferred the ability to consume maltotriose to *S. pastorianus*, several other possibilities have to be considered. Firstly, recent sequencing of *S. pastorianus* strains has revealed the genome complexity of lager-brewing yeasts in which variation and increase in chromosome copy number, a recurrent feature in these strains, has altered gene copy number. Gene duplication is known to promote allelic variation [291] and may have led to the concurrent expression of proteins with properties different from that of the original alleles inherited from the *S. cerevisiae* and *S. eubayanus* ancestors. It is therefore very well possible that *S. pastorianus* harbors *ScAGT1* functional alleles. Secondly, the high-sequence homology between the genomes of

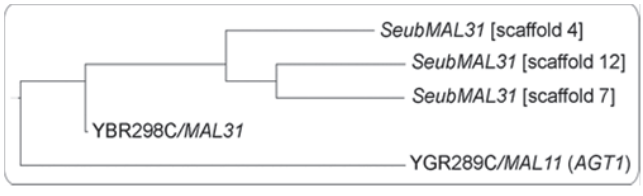


Figure 5. Topographic phylogenetic tree of annotated *S. cerevisiae* and *S. eubayanus MALX1* genes calculated by the neighbour-joining method. Assembled *S. eubayanus* scaffold 4, 7 and 12 were aligned to the S288C reference sequences of *MAL31*/YBR298C and *MAL11*/YGR2289C.

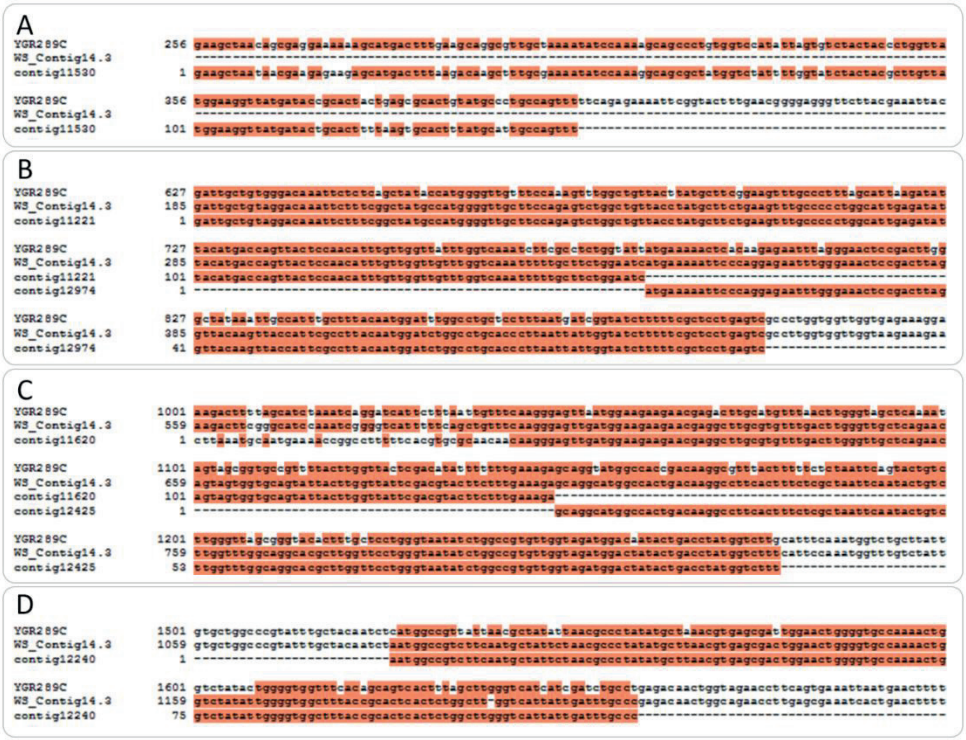


Figure 6. Alignment to *AGT1/MAL11* from *S. cerevisiae* of contigs from the model lager-brewing strain Weihenstephan 34/70 [83] and from the Tibetan *S. eubayanus* strain CDFM21L.1 [108]. *S. cerevisiae AGT1/MAL11*: YGR289C, Weihenstephan 34/70: contig WS14.3. **(A)** CDFM21L.1 contig11530. **(B)** CDFM21L.1 contig11221 and contig12974. **(C)** CDFM21L.1 contig11620 and contig12425. **(D)** CDFM21L.1 contig12240.

S. eubayanus CBS12357 and the *S. eubayanus* subgenome in *S. pastorianus* is not sufficient to conclude that this Patagonian *S. eubayanus* isolate is a direct ancestor of current *S. pastorianus* strains. As recently demonstrated [108, 292], *S. eubayanus* occurs on several continents (i.e. Asia, North and South America). Biodiversity within the *S. eubayanus* species can therefore be expected, and, contrary to the Patagonian isolate, the original *S. eubayanus* parent of *S. pastorianus* may have carried a different set of *MALx1* genes, enabling maltotriose utilization. Indeed, analysis of the highly fragmented genome sequence of the recently identified Tibetan *S. eubayanus* isolate [108] identified six very short contigs (<200 bp) that exhibited better similarity with *MAL11/AGT1* than with *MAL31* (Fig. 6). Out of these six contigs, five completely aligned on the *MAL11/AGT1* nucleotide sequence. 87% of the 106 last nucleotides of the remaining contig (contig11620, 148 bp) could be perfectly aligned to the *MAL11/AGT1* sequence, while the first 42 bases showed poor similarity (16%) with the reference sequence, suggesting that *S. eubayanus MAL11/AGT1* might present structural differences. Additionally, alignment of these short *S. eubayanus* contigs to the *S. pastorianus* Weihenstephan 34/70 contig

(WS-14.3) containing the putative *SeubAGT1* allele [LBYG13187] showed a near-perfect identity (Fig. 6). Out of the six Tibetan *S. eubayanus* contigs, three (contig11221, contig12974, contig12425) were identical to *SeubAGT1* Weihenstephan allele (Fig. 6). As proposed by Bing and co-workers, the Tibetan *S. eubayanus* is more closely related to *S. pastorianus*' ancestor than the strain isolated from Patagonia. The ability of newly isolated American strains and of the Tibetan strains to grow on maltotriose is hitherto unknown [108, 292]. Further mining of genomic diversity in the genus *Saccharomyces*, combined physiological characterization of strains and kinetic characterization of transporters, is required to resolve the origin of maltotriose transport in current brewing strains.

S. cerevisiae × *S. eubayanus* hybrid, a rare example of best parent heterosis

The hybrid displayed an extended range of growth environments as compared to its parents. From its *S. cerevisiae* parent, the hybrid inherited the ability to grow at 35°C and to consume maltotriose, while its *S. eubayanus* subgenome endowed the hybrid with a substantially faster and more efficient growth at low temperature. At extreme temperatures of 8 and 35°C, the hybrid was not able to perform as well as its best parent as shown by the intermediate growth rate between that of the parents. However, growth at various temperatures revealed a striking transgression for temperature ranges between 20 and 30°C at which the hybrid substantially and significantly outperformed the best parent. This best parent heterosis was even more pronounced when oligosaccharides were used as carbon source. Maltose is a dimer of glucose and its utilization requires a transporter and a maltase that will hydrolyze maltose into glucose. It has recently been shown that *S. pastorianus* harbors different *MAL11/AGT1* genes with different temperature sensitivities [199]. The maltose transporters of the more cryotolerant *S. eubayanus* may similarly perform better than *S. cerevisiae*'s transporters at low temperature.

The presented hybrid is just one sample of the genetic and phenotypic landscape. However, in view of the rare incidence of best parent heterosis [293-295], it is highly likely that this feature is shared by *S. cerevisiae* × *S. eubayanus* offspring. A surprising aspect of the reticulate evolution of *S. pastorianus* is that *S. eubayanus* has hitherto not been isolated in Europe, the continent where lager-brewing yeast first appeared and evolved [81]. While encounters between *S. cerevisiae* and *S. eubayanus* may have been scarce due to their geographical distribution, the best parent heterosis of *S. cerevisiae* × *S. eubayanus* hybrids may explain why they have become a predominant brewing yeast. Indeed, the strong selective advantage of this hybrid in the brewing environment may have been decisive in its colonization of wort and subsequent domestication.

As could be anticipated, the physiology of the interspecific hybrid was not identical to that of the lager-brewing yeast, even if the two strains shared some interesting features. It would be particularly interesting to investigate the physiology of a range of hybrids constructed from different parents, and more especially from ale *S. cerevisiae*'s strains and Chinese *S. eubayanus* isolates, and with different ploidies (tri- and tetraploids) that may better reflect the genomes of the hybrid ancestors of the modern lager-brewing yeast. A recent study has demonstrated the power of directed evolution of interspecific hybrid to investigate the genomic fate of newly formed interspecific hybrids [252]. Similarly directed evolution of *S. cerevisiae* × *S. eubayanus* hybrids in brewing-like environment should prove pivotal in understanding the evolutionary path and forces that have shaped the lager-brewing yeast genome.

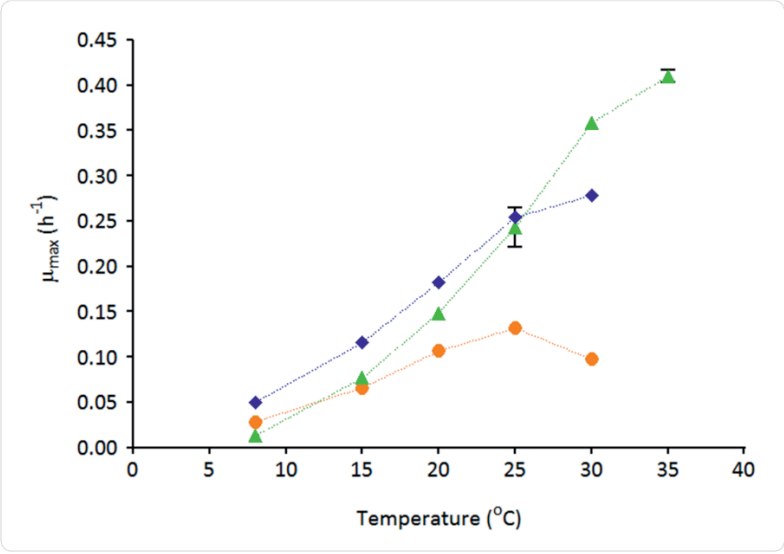
As a final remark, it is worth mentioning the recent work of Krogerus and co-workers that further reinforces our observations and complements the present study [112].

Acknowledgements

We thank Niels Kuijpers, Daniel Solis-Escalante, Mathijs Martens and Matthijs Niemeijer for their experimental support and Peter Verheijen for helping with statistical analysis of the data. We thank Dr Feng-Yan Bai for sharing the raw sequencing data of the *S. eubayanus* strain CDFM21L.1 (BioProject PRJNA254367).

This project was carried out within the research program of the Netherlands Consortium for Systems Biology, which is sponsored by the Netherlands Genomics Initiative. AB is funded by the Seventh Framework Programme of the European Union in the frame of the SP3 people support for training and career development of researchers (Marie Curie), Networks for Initial Training (PITN-GA-2013 ITN-2013-606795) YeastCell.

Supplementary Material



Supplemental Figure S2. Temperature tolerance of diploids *Saccharomyces kudriavzevii* CR85, *S. cerevisiae* CEN.PK122 and *S. eubayanus* CBS12357. The maximum specific growth rate (μ_{max}) of *S. cerevisiae* CEN.PK122 (green), *S. eubayanus* CBS12357 (blue) and *S. kudriavzevii* (orange) at 8, 15, 20, 25, 30 and 35°C was determined in anaerobic SBR's using SM-Glu. μ_{max} was calculated during the second batch at each temperature from continuous CO_2 measurements in the offgas. The data represent the average and standard error of the mean of independent duplicate cultures.

Supplementary Table S1. Biomass specific rates of *S. cerevisiae*, *S. eubayanus*, the *S.c. x S.e.* interspecific hybrid and *S. pastorianus* grown in SBR in SM-Glu at 12 and 30 °C. Average of two independent culture replicates and standard error of the mean are shown

	S. cerevisiae CEN.PK122		*S. eubayanus* CBS12357		*S. cerevisiae x S. eubayanus* IMS0408		*S. pastorianus CBS1483*	
	30°C	12 °C	30°C	12 °C	30°C	12 °C	30°C	12 °C
μ_{max} (h^{-1})	0.329±0.006	0.039±0,001	0.255±0.005	0.079±0.001	0.370±0.009	0.063±0.001	0.217±0.011	0.035±0.000
Biomass specific rates (mmol·g $DW^{-1}.h^{-1}$)								
Glucose	-3.8±0.2	-20.3±0.5	-5.2±0.0	-15.6±0.3	-4.4±0.1	-21.6±0.7	-2.4±0.1	-13.2±1.0
Ethanol	4.8±0.2	28.3±0.7	7.4±0.1	21.7±0.4	6.4±0.1	29.6±1.1	3.5±0.1	19.5±1.5
Glycerol	0.69±0.03	3.92±0.10	0.90±0.02	3.09±0.09	0.91±0.02	4.31±0.12	0.40±0.02	2.51±0.13
Pyruvate	0.0078±0.0003	0.124±0.003	0.0265±0.0005	0.1315±0.0035	0.0123±0.0003	0.1649±0.0075	0.0024±0.0002	0.1138±0.0086
Succinate	0.0124±0.001	0.154±0.020	0.0304±0.001	0.1058±0.0033	0.0212±0.0005	0.1769±0.03	0.0245±0.0007	0.0633±0.0046
Lactate	0.026±0.001	0.26±0.01	0.081±0.001	0.284±0.009	0.072±0.002	0.327±0.01	0.016±0.001	0.217±0.016
Acetate	0.19±0.01	0.48±0.02	0.062±0.003	0.265±0.018	0.167±0.004	0.439±0.024	n.d. [a]	0.147±0.015

[a] The produced acetate was reconsumed by *S. pastorianus* before the end of the fermentation, thereby biasing calculation of the acetate yield.

Chapter 4: Evolutionary engineering in chemostat cultures for improved maltotriose fermentation kinetics in *Saccharomyces pastorianus* lager brewing yeast

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Essentially as published in **Frontiers in Microbiology** 2017, 8:1698 (https://doi.org/10.3389/fmicb.2017.01690)

Abstract

The lager brewing yeast *Saccharomyces pastorianus*, an interspecies hybrid of *S. eubayanus* and *S. cerevisiae*, ferments maltotriose, maltose, sucrose, glucose and fructose in wort to ethanol and carbon dioxide. Complete and timely conversion ('attenuation') of maltotriose by industrial *S. pastorianus* strains is a key requirement for process intensification. This study explores a new evolutionary engineering strategy for improving maltotriose fermentation kinetics. Prolonged carbon-limited, anaerobic chemostat cultivation of the reference strain *S. pastorianus* CBS1483 on a maltotriose-enriched sugar mixture was used to select for spontaneous mutants with improved affinity for maltotriose. Evolved populations exhibited an up to five-fold lower residual maltotriose concentration and a higher ethanol concentration than the parental strain. Uptake studies with ^{14}C-labelled sugars revealed an up to 4.75-fold higher transport capacity for maltotriose in evolved strains. In laboratory batch cultures on wort, evolved strains showed improved attenuation and higher ethanol concentrations. These improvements were also observed in pilot fermentations at 1000-L scale with high-gravity wort. Although the evolved strain exhibited multiple chromosomal copy number changes, analysis of beer made from pilot fermentations showed no negative effects on flavour compound profiles. These results demonstrate the potential of evolutionary engineering for strain improvement of hybrid, alloploid brewing strains.

Introduction

Over the last decades, the global beer industry has grown to reach a global volume of 193 billion litres (data for 2015, https://www.statista.com). Lager beer accounts for 89% of this volume, making it the most-produced fermented beverage. The microbial work horse of lager fermentation is *Saccharomyces pastorianus*, a natural hybrid of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* [74, 83] that has been domesticated in Europe since the late Middle Ages [1]. Two main *S. pastorianus* lineages emerged from this domestication, commonly referred to as "Saaz" (Group 1) and "Frohberg" (Group 2) strains [76]. These groups display distinctive phenotypic characteristics. Saaz yeasts generally exhibit a higher cold tolerance and stronger tendency to flocculate, while Frohberg strains exhibit faster fermentation and maltotriose conversion [80, 81]. These characteristics coincide with differences in their genome composition. In Saaz strains, a sizeable fraction of the *S. cerevisiae* subgenome (e.g. chromosomes (CHR) VI and VIII as well as parts of IV, XIII and XV) is absent, whereas modern industrial Frohberg yeasts retain a near-complete set of chromosomes from both parents [77, 82, 84]. Origins of these two lineages is still a matter of debate, some studies have advocated for two different hybridization histories [76, 78, 298] whereas others privileged a common primary hybridization event followed by divergent evolutionary paths [78].

The growing popularity of lager beer provided the brewing industry with incentives to intensify the industrial fermentation process [299, 300]. This intensification was primarily achieved by increasing the gravity of wort. Gravity, in the context of brewing, indicates the density of the wort relative to that of water and predominantly depends on the sugar content [301]. Current industrial lager fermentations are performed at concentrations of fermentable sugars ranging from 150 to 200 g L^{-1} (16° to 20° Plato gravity equivalent). High-gravity fermentation improves process economics as well as sustainability [68]. Malted barley wort, the substrate for lager brewing, contains five main fermentable sugars: the monosaccharides glucose and fructose, the disaccharides maltose and sucrose and the trisaccharide maltotriose. Wort dextrins, which are glucose chains with more than three moieties, contribute to wort gravity but cannot be fermented by *S. pastorianus* strains [302].

Wort fermentation by *S. pastorianus* strains is usually initiated by a fast, preferential consumption of glucose and fructose. In *Saccharomyces* yeasts, these sugars are transported across the plasma membrane by a large set of hexose facilitator (HXT) transporters. The genome of the laboratory model *S. cerevisiae*, encompasses 23 *HXT* genes [303]. Hxt transporters in *S. eubayanus*, the other contributor to the hybrid genome of *S. pastorianus*, have not yet been characterized in detail. However, its genome harbours nine genes annotated as *HXT* orthologs [34, 75]. In addition to these energy-independent hexose facilitators, a fructose/H^+ symporter, inherited from *S. eubayanus*, has been

identified in *S. pastorianus*, but not in *S. cerevisiae* [258, 304]. Maltose and maltotriose represent over 70 % of the total sugar content of wort [23]. These oligosaccharides are typically taken up by *S. pastorianus* strains after depletion of glucose and fructose [301, 305].

In *Saccharomyces* yeasts, maltose is transported across the plasma membrane by proton symporters [119, 195, 232, 306]. Maltose-proton symport is energized by the plasma-membrane proton-motive force, which is maintained by the proton-pumping plasma-membrane ATPase Pma1 [195, 231]. Once inside the cell, maltose is hydrolysed by α-glucosidase (maltase) into two glucose molecules [121]. Maltotriose is assumed to be assimilated through the same system [123, 131, 307]. In *S. cerevisiae*, the genes involved in maltose metabolism are clustered in *MAL* loci, which are located in the subtelomeric regions of multiple chromosomes. A *MAL* locus ("*MALx*") includes three genes, encoding an α-glucoside transporter (*MALx1*), an α-glucoside hydrolase (maltase, *MALx2*) and a transcriptional regulator of gene cluster (*MALx3*). The number and identity of *MAL* loci is highly strain dependent [84, 93, 308], with five loci (*MAL1, MAL2, MAL3, MAL4 and MAL6*) having been identified and characterized in different strains [130, 191-194, 309]. All *MALx1* genes share high sequence similarity (>95% at nucleotide level), except for *MAL11* (also referred to as *AGT1*), whose DNA sequence displays only 57% identity to the other four *MALx1* transporter genes [143]. In contrast to other *MALx1*-encoded transporters, Agt1 can efficiently transport α-glucosides other than maltose, such as trehalose [196], sucrose [197, 198] and, importantly, maltotriose [129]. The genome of S. *eubayanus* CBS12357 harbours four putative maltose transporter genes (*SeMALT1*; *SeMALT2*, *SeMALT3* and *SeMALT4*) [75] which, however, have not yet been functionally analysed. However, none of them appear to transport maltotriose since *S. eubayanus* CBS12357 is unable to grow on this trisaccharide [34].

Maltose and maltotriose transport capacities in *S. pastorianus* cannot be described as a simple combination of transporters encoded by the genomes of current *S. cerevisiae* and *S. eubayanus* strains. While most *S. cerevisiae MAL* loci can be identified in *S. pastorianus* genomes, significant strain differences occur. For example, *AGT1* alleles in the *S. cerevisiae* subgenomes of the *S. pastorianus* strains Weihenstephan 34/70 [83], CBS1483 [84] and A15 [138] carry a nucleotide insertion that interrupts their reading frames, causing a loss of function. A different, complete *AGT1* allele in these strains has been proposed to be derived from the *S. eubayanus* parental genome [83, 145], although no *AGT1* ortholog occurs in the genome of *S. eubayanus* CBS12357 [75]. Additionally, *MTT1/MTY1* gene, which shows 90% identity with *MALX1*, has been identified in *S. pastorianus* strains and proposed to be involved in maltose and maltotriose transport at low temperature [131, 133, 134]. In total, maltose and maltotriose transport in *S. pastorianus* may involve up to ten different transporter genes which, taking into account the aneuploidy of its alloploid genome, could represent over 25 alleles in a single strain.

While most *S. pastorianus* strains rapidly ferment maltose, kinetics of maltotriose fermentation are typically much slower, resulting in extended fermentation

process times and, in many cases, incomplete fermentation of this sugar [80, 122, 159, 310]. In addition to the genetic complexity of maltose and maltotriose metabolism in *S. pastorianus*, consumer acceptance issues preclude the use of targeted metabolic engineering approaches for improving sugar fermentation kinetics. Evolutionary engineering, also referred as adaptive laboratory evolution (ALE) [311], uses laboratory evolution to improve industrially relevant phenotypic characteristics and is a powerful non-GM approach for strain improvement [190, 312]. Moreover, resequencing of the resulting evolved genomes can provide important insights into the genetic basis for the acquired improved performance [313].

The aim of the present study was to test an evolutionary engineering strategy for obtaining *S. pastorianus* strains with improved sugar fermentation kinetics and, in particular, with a faster conversion of maltotriose at the end of fermentation. To maintain a constant selective pressure for spontaneous mutants with an improved affinity for maltotriose, the model strain *S. pastorianus* CBS1483 (group II, Frohberg) was grown in carbon-limited chemostat cultures [233] on a maltotriose-enriched sugar. After prolonged cultivation, single-cell lines were isolated and characterised in different culture systems, from bench-top fermenters up to 1000 L pilot fermentation scale. Additionally, the performance of a selected strain was evaluated based on the quality of the bottled beer produced. Special attention was focused on the question of whether and to what extent laboratory evolution of an alloploid brewing strain for improved sugar fermentation kinetics affected profiles of flavour compounds in the final product.

Material and Methods

Strains and maintenance

Yeast strains used in this study are listed in Table 1. Stock cultures were grown in YPD (10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone and 20 g L^{-1} glucose) until stationary phase, supplemented with sterile glycerol (final concentration 30% (v/v)) and stored at -80 ^{0}C as 1 ml aliquots until further use.

Table 1: *Saccharomyces* strains used in this study.

Strain name	Relevant genotype/ description	Reference
S. cerevisiae CEN.PK113-7D	*Mata SUC2 MAL2-8c*	[267]
S. eubayanus CBS12357	Isolated in Patagonia	[74] CBS database[a]
S. pastorianus CBS1483	Group II brewer's yeast, Heineken's bottom yeast, July 1927	CBS database[a]
S. pastorianus IMS0493	Evolved CBS1483	This study
S. pastorianus IMS0495	Evolved CBS1483	This study
S. pastorianus IMS0507	Evolved CBS1483	This study
S. pastorianus IMS0508	Evolved CBS1483	This study

[a] http://www.westerdijkinstitute.nl/collections/.

Media

Chemostat and bioreactor batch cultures were grown on synthetic medium (SM) containing 3.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4$, 7 H_2O, 1 mL L^{-1} trace element solution, and 1 mL L^{-1} vitamin solution [314]. A sugar mixture (Dried Glucose syrup C plus 01987, Cargill, Haubourdin, France; sugar content (w/w): 2.5 % glucose, 28.0 % maltose, 42.0 % maltotriose, 26.4 % higher saccharides) was added to SM to a final concentration of fermentable sugars of 20 g L^{-1} (SM-Mix). For cultivation on solid medium, SM or SM-Mix was supplemented with 2 % (w/v) agar. SM used for anaerobic bioreactor cultivation was supplemented with ergosterol and Tween-80 (0.01 g L^{-1} and 0.42 g L^{-1}, respectively; [314]) and with 0.15 g L^{-1} of antifoam C (Sigma-Aldrich, Zwijndrecht, The Netherlands). In all cases, the pH was adjusted to 5.0 with 1 M HCl. For assays of maltose and maltotriose transport, yeast cells were pregrown in YPM (10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone and 20 g L^{-1} maltose).

For propagation and cultivation in E.B.C. (European Brewery Convention) tall-tubes [315], strains were grown in aerated (18 ppm DO) 15 ° Plato industrial wort. Wort was produced from barley and wheat malt at the VTT Pilot Brewery (VTT Technical Research Centre of Finland Ltd, Espoo), and contained an extract of 15.0 °Plato (62 g L^{-1} maltose, 22 g L^{-1} maltotriose, 16 g L^{-1} glucose, and 4.6 g L^{-1} fructose) and a free amino nitrogen (FAN) content of 269 mg L^{-1} [80]. Pilot fermentation experiments were carried out in 1300 L stainless steel tanks filled with 1000 L of 15 ° Plato industrial wort. Prior to inoculation, the wort was aerated with 25 ppm of O_2 and supplemented with zinc to a final concentration of 1.2 mg L^{-1}. The culture was pitched with 1.0 x 10^7 cells mL^{-1}. Precultures were prepared by sequentially propagating cells from frozen stocks, into 50 mL, 500 mL, 12 L and and 1000 L pre-cultures on wort. All inoculum preparation stages were carried out at 20 °C, except the last stage performed at 15 °C.

Culture conditions

Carbon-limited chemostat cultures

Laboratory evolution experiments were performed in four independent anaerobic chemostat cultures, grown on SM-mix at a dilution rate of 0.05 h^{-1}. Two chemostat cultures (7L2 and 7R1) were grown in 2 L Applikon bioreactors (Applikon, Delft, The Netherlands), with a 1 L working volume of SM-Mix. The two other chemostat cultures (M1L and M2L) were performed in Multifors benchtop bioreactors (Infors, Velp, The Netherlands) with a working volume of 100 mL. Nitrogen gas (<10 ppm oxygen) was sparged through the cultures at 0.5 L min^{-1} to ensure anaerobic conditions. Reactors were equipped with Norprene tubing (Saint-Gobain Performance Plastics, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, The Netherlands) to minimize oxygen diffusion. Culture pH was controlled at 5.0 by automated addition of 2.0 M KOH, the temperature

was controlled at 16 °C and stirrer speed was set at 800 rpm. Cultures were regularly sampled for analysis of OD_{660}, biomass dry weight (only for 1-L working volume cultures) and metabolites (glucose, maltose, maltotriose, glycerol and ethanol). Cultivation was continued until residual maltotriose concentrations stabilized. Chemostat cultures for comparing evolved isolates and the parental strain *S. pastorianus* CBS1483 were performed in 1 L working volume fermenters under the conditions described above. Cultures were analysed when biomass concentrations in samples taken at 4, 6 and 9 volume changes after the onset of continuous cultivation varied by less than 2 % [189, 233]. Precultures were grown in 100-mL shake-flask at 20 °C on SM-Mix medium and all chemostat cultures were inoculated at a cell density of 0.2 OD_{660} U L^{-1}.

Bioreactor and tall-tube batch cultivation

Bioreactor batch cultures were grown in 2-L Applikon bioreactors (Applikon) with a 1-L working volume, equipped as described for chemostat cultivation, on SM-Mix. The cultures were grown at 15 or 20°C, pH was controlled at 5.0 and 100 mL inoculum cultures were grown at 20°C in 500 mL shake-flask on SM-Mix to an OD_{660} ranged between 10 and 12. The cultures were inoculated with 10 mL inoculum. Independent duplicate cultures were analysed for each combination of strain and growth conditions.

For characterization of evolved isolates in wort, batch cultivation was performed at 15 °C in 2-L cylindro-conical stainless-steel tall-tube fermentation vessels containing 1.9 L of wort. The wort medium was oxygenated to 18 mg L^{-1} before inoculation. Tall-tube experiments were preceded by a biomass propagation step on wort in the same set-up. When, at the end of this first fermentation, ethanol concentrations had remained constant for 24 h, yeast biomass was harvested by centrifugation and washed twice with sterile MilliQ-filtered water and resuspended to give a 20% (w/v) yeast suspension. The main fermentation was inoculated at a concentration of 5 $g_{wet\ biomass}$ L^{-1} [80].

Pilot-scale fermentation experiments

The evolved strain *S. pastorianus* IMS0493 and parental strain CBS1483 were grown under industrial conditions. Independent duplicate fermentations were performed for both strains, in 1300 L non-stirred stainless steel vessels containing 1000 L of aerated wort (25 ppm O_2) at a gravity of 15.0 ° Plato [316]. Wort was supplemented with zinc to a final concentration of 1.2 mg L^{-1}. The main fermentation was inoculated at a cell density of 1.10^6 cells mL^{-1} and an initial temperature of 8 °C, which was allowed to freely rise to 11 °C during fermentation. When the gravity had decreased to 6.5 °Plato, the temperature was increased to 14°C for a “ruh” phase [316]. As diacetyl levels dropped below 20 ppb, the broth was cooled to 0°C within 14 h and lagered for 5 days. The beer was then filtered on a 0.4 µm membrane bottled in 30 cl capped bottles and pasteurized. Biomass from the final 1000 L propagation step was harvested and stored in a cooled

storage tank. Samples from pilot fermentations were taken at 24 h intervals. Bottled beers were also sampled and analysed.

Isolation of single cell lines from evolution experiments

Single cell lines were isolated from prolonged chemostat experiments by plating on solid SM-Mix and incubating plates anaerobically at 20°C. After three consecutive restreaks, one single-colony isolate from each evolution experiment was selected and characterized [317].

Analytical methods

Optical density of cultures (OD_{660}) was determined with a Libra S11 spectrophotometer (Biochrom, Cambridge, UK) at 660 nm. Biomass dry weight was determined by filtering duplicate 10 mL culture samples over preweighed nitrocellulose filters with a pore size of 0.45 µm. Filters were washed with 10 mL demineralized water, dried in a microwave oven (20 min at 350 W) and reweighed. Off gas of bioreactor fermentations was cooled to 4 ^{0}C in a condenser and CO_2 concentration was continuously monitored with an NGA 2000 analyser (Rosemount Analytical, Orrville, OH). For metabolite analysis, bioreactor culture samples were centrifuged and supernatants were analysed with a Waters Alliance 2690 HPLC (Waters Co., Milford, MA) containing a Waters 2410 refractive-index detector, a Waters 2487 UV detector, and a Bio-Rad HPX-87H column (Bio-Rad, Hercules, CA) which was equilibrated and eluted with 0.5 g L^{-1} H_2SO_4 at 60°C at a 0.6 mL min^{-1} flow rate. Sugar concentrations in samples from tall tube fermentations were analysed with a Waters HPLC with a Waters 2695 separation module and a Waters 2414 differential refractometer (Waters Co., Milford, MA) and an Aminex HPX-87H organic acid analysis column (Bio-Rad, Hercules, CA) equilibrated at 55 °C and eluted with 5 mM H2SO4 in water at a 0.3 ml min^{-1} flow rate.

Cell count and viability were determined using a NucleoCounter® YC-100™ (Chemometec A/S, Allerod, Denmark) according to the manufacturer's recommendations. The specific gravity, alcohol content and pH in pilot-scale experiments and bottled beer were analysed after filtration using an Anton Paar density meter (Anton Paa r GmbH, Graz, Austria). Concentration of fermentable sugars and volatile compounds (acetaldehyde, acetone, ethylformate, ethylacetate, methanol, ethylpropanoate, propanol, isobutanol, isoamylacetate, ethylcaproate, dimethyl sulfide, diacetyl and 2,3-pentanedione) in these samples were determined by ultra-performance liquid chromatography (UPLC) (Waters Co) and gas chromatography, respectively.

To determine if two sets of data were significantly different from each other unpaired two-sample Student's t-test was used in Prism 4 (version 4.03) (Graphpad software Inc., La Jolla, CA).

Analysis of sugar-uptake kinetics

Sugar-uptake kinetics by yeast cell suspensions were determined with [^{14}C] labelled maltose and maltotriose [0.1 mCi mL^{-1}] (American Radiolabelled Chemicals, St Louis, MO). Prior to use ^{14}C-labelled maltotriose was treated to remove impurities as described previously in [132]. Transport rates were calculated from the radioactivity remaining inside the yeast cells after washing [318]. Yeast strains were pregrown in 100 mL YPM at room temperature and harvested at an OD_{600} between 3 and 7. After centrifugation, yeast pellets were washed with ice-cold water, followed by washing with ice-cold 0.1 M tartrate-Tris buffer (pH 4.2) and resuspended to a cell concentration of 200 mg yeast wet weight mL^{-1} in the same buffer. Yeast suspensions were equilibrated to the 20°C assay temperature in a water bath. Experiments for maltose and maltotriose uptake rates were performed at different sugar concentration ranged from 1 to 25 mM. Sugar uptake was stopped after 60 s by addition of 5 mL ice-cold water and immediate filtration through a HVLP membrane (0,2 µm) (Millipore, Merck Life Science, Espoo, Finland). After rinsing the membrane with another 5 mL of ice-cold water, it was transferred to a scintillation cocktail (Optiphase Hisafe 3, PerkinElmer, Waltham) and the radioactivity was counted in a scintillation counter (Tri-Carb 2810TR Low Activity Liquid Scintillation Analyzer, PerkinElmer). K_m and V_{max} were derived from Eadie-Hofstee plots fitting of the measured rates [319]. The statistical significance of observed differences between CBS1483 and IMS0493 strains was assessed by unpaired two-sample Student's t-test was used in Prism 4 (version 4.03) (Graphpad software Inc., La Jolla, CA).

Genome sequencing

DNA was isolated from strains *S. pastorianus* IMS0493 and CBS1483 as previously described in [84]. Whole-genome sequencing was performed by Novogene (HK) Company Limited (Hong Kong, China). A DNA library was produced with the TruSeq DNA PCR-Free Library Preparation kit (Illumina, San Diego, CA). Paired-end libraries with 354 and 370 bp inserts were prepared for strains CBS1483 and IMS0493, respectively, and sequenced with an Illumina HiSeq2500 sequencer (Illumina). A total of 4.3 Gb and 4.6 Gb of 150-bp paired-end fragments were generated for IMS0493 and CBS1483 respectively. Chromosome copy numbers were estimated using the Magnolya software tool [188, 267]. For visualization purposes, assembled contigs and their copy number were mapped on an illumina reads based-assembly of *S. pastorianus* CBS1483 (ASM80546v1) [84]. The copy number estimation of individual maltose transporter genes was performed by concatenating sequences of *SeMALT1* (GenBank Accession number: XM_018363333.1), *SeMALT2* (XM_018364778.1), *SeMALT3* (XM_018367005), *SeMALT4* (XM_018368187.1), *ScMAL11* (AJ012752.1), *ScMAL31* (NM_001178646.1), *ScMAL32* (NM_001178647.3), *SNF5*/YBR289W (Z36158.1), *MSH3*/YCR092C (M96250.1),

MES1/YGR264C (Z73049.1), *SeBRN1*/SeYBL097W (XM_018363344.1) *SePDA1*/SeYER178W (XM_018364769.1) *SeATF1*/SeYOR377W (XM_), *SeGTR1*/SeYML121W (XM_018367018.1), *SePLC1*/SeYPL268W (XM_018368196.1) into an artificial contig onto 30.10^{+6} sequencing reads from IMS0493 or CBS1483 were mapped using Burrows Wheeler Aligner BWA [216] with default parameters. For each alignment 100bp-coverage windows were calculated. For each gene 100bp coverage windows data were collected and CBS1483 and IMS0493 data were statistically assessed for significant difference using unpaired two-sample Student's t-test was used in Prism 4 (version 4.03) (Graphpad software Inc., La Jolla, CA). Per gene average and standard deviation were calculated and plotted.

The raw sequencing data of strains IMSS0493, IMS0495 and IMS0508 are searchable at NCBI Entrez (http://www.ncbi.nlm.nih.gov/) under BioProject number PRJNA393253. The data of the parental strain CBS1483 can be found in BioProject PRJNA266750 (accession number SRP049726) [84].

Results

Suboptimal maltotriose fermentation by *S. pastorianus* CBS1483

In brewing, the term 'attenuation' describes the extent to which brewing yeast completely converts wort extract into ethanol, CO_2, yeast biomass and flavour compounds. Optimal attenuation requires efficient consumption of glucose, fructose, maltose as well as maltotriose and is a highly sought-after phenotypic trait in industrial *S. pastorianus* strains. Despite their history of domestication in wort, many lager yeasts exhibit incomplete or slow fermentation of the two major wort ?-oligosaccharides, maltose and maltotriose.

Since preliminary results indicated that the lager-brewing strain *S. pastorianus* CBS1483 could be an interesting model to study low attenuation due to suboptimal maltotriose fermentation kinetics in lager brewing strains, its growth and fermentation performance in 15 ^{0}Plato wort was quantitatively analysed in cylindrical 2-L stainless steel vessels at 15 ^{0}C. Indeed, after 15 days of fermentation, residual concentrations of maltose and maltotriose remained at 3.2 ± 0.1 g L^{-1} and 14.2 ± 0.1 g L^{-1}, respectively (Figure 1A). In these experiments, the fermentable sugars in wort were sequentially consumed. Glucose was fermented first, followed by maltose, while maltotriose consumption only began after 30 h. To investigate whether the incomplete utilisation of maltose and maltotriose was caused by a limiting amount of available nitrogen in the wort, additional experiments with *S. pastorianus* CBS1483 were performed in stirred bioreactors on synthetic medium with excess nitrogen and lower overall sugar concentration (20 g L^{-1} of a sugar mixture containing 2.5 % glucose, 28.0 % maltose, 42.0 % maltotriose and 26.4% higher dextrins). As observed in the wort fermentations (Figure

1A), a sequential utilization of the fermentable sugars was observed (Figures 1B, and S1). Maltotriose consumption slowed down severely when its concentration reached ca. 1 g L^{-1}, and 0.25 g L^{-1} maltotriose was left at the end of fermentation.

These data indicated that, irrespective of the initial sugar concentration and nitrogen source availability, *S. pastorianus* CBS1483 could not completely consume maltotriose. This phenotype was stronger under the higher gravity fermentation conditions representative of industrial brewing. It was therefore decided to use strain CBS1483 as a model to explore an evolutionary engineering strategy for improving the kinetics of maltotriose fermentation.

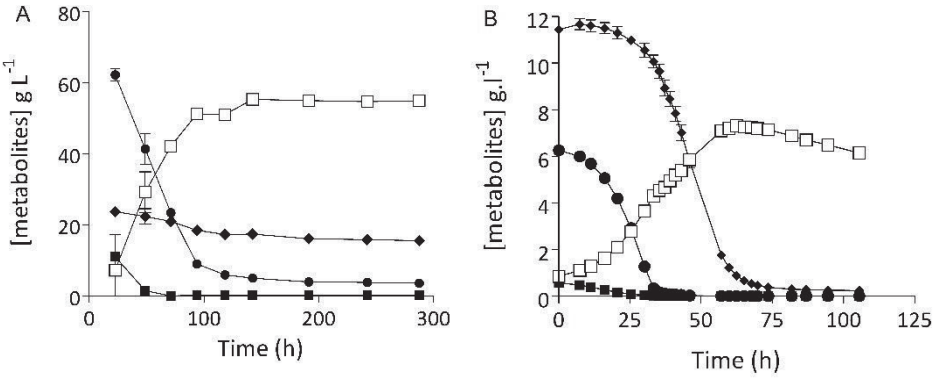

Figure 1: *S. pastorianus* CBS1483 cannot complete wort α-oligosaccharide fermentation in anaerobic batch cultures: Metabolites determined (by HPLC) from filtered supernatant from *S. pastorianus* CBS1483 cultures. A) static fermentation in 2-L cylindrical fermentation tubes in 15 ^{0} Plato wort at 15 ^{0}C; B) stirred 2-L laboratory bioreactor cultures grown on synthetic medium (SM-Mix, containing 2.5% glucose, 28.0% maltose, 42.0% maltotriose and 26.4% higher dextrin) at 16^{0}C. (■) glucose, (●) maltose, (♦) maltotriose, (□) ethanol. Graph shows average of biological independent duplicate fermentations ± standard error.

Evolutionary engineering of *S. pastorianus* CBS1483 for improved growth substrate affinity

The batch cultivation experiments on wort and on maltotriose-enriched sugar mixtures (Figure 1) clearly indicated suboptimal kinetics of maltotriose fermentation in *S. pastorianus* CBS1483. In particular, a deceleration of fermentation at low maltotriose concentrations contributed to a poor attenuation in these mixed-substrate cultures. The ability of microorganisms to maintain high biomass-specific substrate conversion rates (q_s) at growth-limiting concentrations of a substrate (C_s), is referred to as the affinity for that substrate. When growth kinetics obey the Monod equation ($q_s = q_{s,max} (C_s/C_s + K_s)$), affinity can be defined as $q_{s,max}/K_S$ [320], in which $q_{s,max}$ is the maximum biomass-substrate uptake rate and K_s is the substrate concentration at which q_s equals 50 % of $q_{s,max}$. Nutrient-limited chemostat cultivation confers a strong selective advantage to

spontaneous mutants with an improved affinity for the growth limiting nutrient. During prolonged chemostat cultivation, a gradual 'take over' of cultures by mutants with an improved affinity for the growth-limiting nutrient is often reflected by a progressive decrease of its residual concentration in the cultures (for examples see [189, 233]). To explore whether this concept can be applied to improve the affinity and fermentation kinetics of *S. pastorianus* CBS1483 for maltotriose, four independent chemostat experiments were performed. In these carbon-limited chemostat cultures, which were grown at a dilution rate of 0.05 h^{-1} and at 16 °C, the carbon source consisted of a sugar mixture comprising 2.5 % glucose, 28.0 % maltose, 42.0 % maltotriose and 26.4 % higher saccharides (w/w). Continuous cultivation was started when, in an initial batch cultivation stage on the same sugar mixture, maltotriose was the sole remaining carbon source in the bioreactor.

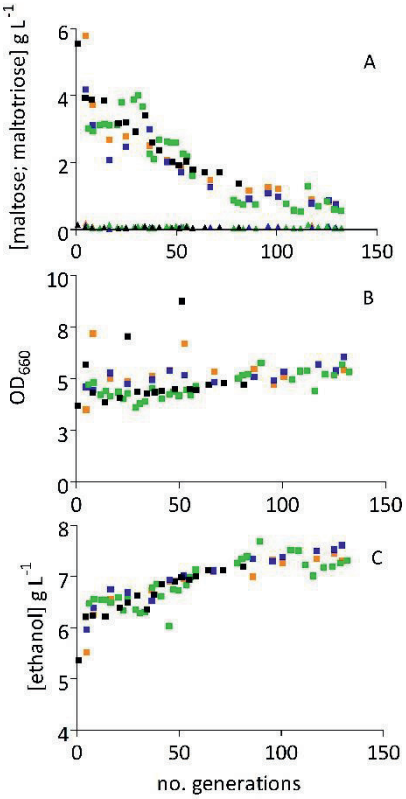

Figure 2: Evolutionary engineering of *S. pastorianus* CBS1483 in chemostat cultures. Graphs A, B, and C display data from four independent evolution experiments in anaerobic, carbon- limited chemostat cultures, grown at 16 °C on SM-Mix medium. Bioreactor working volumes were 100 mL in replicate evolution experiments M1L and M2L and 1 L in replicate experiments 7L2 and 7R1. **A-** residual extracellular maltose (▲) and maltotriose (■) concentrations. **B-** OD measured at 660 nm (■). **C-** Ethanol concentrations (■) during chemostat cultivation. Evolution experiment M1L is represented in blue, M2L in orange, 7R in green and 7L in black.

After 5 volume changes, concentrations of glucose and maltose in the four chemostat cultures were below detection level (< 0.2 g L^{-1} and < 0.1 g L^{-1}, respectively), while maltotriose concentrations had not decreased below 3.0 g L^{-1} (Figure 2A). After approximately 30 generations, the residual maltotriose concentration in all four chemostat experiments gradually decreased until, after 80 generations, a reduction of about 70% was reached. In chemostat experiment 7R (Figure 2, green symbols) the maltotriose concentration decreased from 3.0 g L^{-1} at the beginning of the culture to 0.56 g L^{-1} after 132 generations (Figure 2A). The higher utilization of maltotriose at the end of this fermentation experiment was

accompanied by increases of the biomass and the ethanol concentrations by 17 % (Figure 2B) and 13 % (Figure 2C) respectively.

Single colony isolate IMS0493 expresses an improved phenotype representative of the final evolved culture

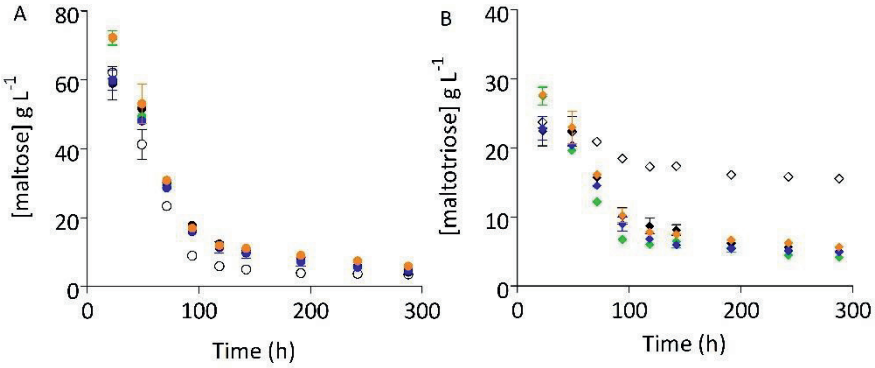

Figure 3: Improved maltotriose consumption of four independent single-colony isolates. Four single-colony isolates (IMS0508, IMS0507, IMS0493 and IMS0495) and their common parental strain *S. pastorianus* CBS1483 were grown in 2 L cylindrical fermentation tubes on 15 ^{0} Plato wort at 15 ^{0}C without mixing. Concentrations of the α-oligosaccharides maltose (A) and maltotriose (B) were measured by HPLC. The data presented show average ± mean deviation of independent duplicate fermentation experiments. Strain IMS0493 is shown in green, IMS0495 in black, IMS0507 in blue and IMS508 in orange. The parental strain CBS1483 is depicted with open circles. Student's t-test analysis indicated significant improvement of maltotriose consumption in all four isolates (^{IMS0493}p-value = 0.0011, ^{IMS0495}p-value = 0.0132, ^{IMS0507}p-value = 0.0057, ^{IMS0508}p-value = 0.0438).

After 130 generations of selective chemostat cultivation on a sugar mixture, the fermentation performance of the evolved cultures could either reflect the characteristics of a mixed population or of a single, dominant enriched clonal population. To investigate whether pure cultures isolated from the evolution experiments exhibited an improved fermentation performance, single colonies were isolated from each evolution experiment. Four of these strains were grown on 15 ^{o} Plato wort in cylindrical 2 L stainless-steel tall tubes, operated at 15 ^{0}C. Single-colony isolates IMS0493, IMS0495, IMS0407 and IMS0508 clearly improved fermentation kinetics relative to their common parental strain CBS1483. In particular, residual maltotriose concentrations were significantly 2.8 to 3.7-fold lower (Student's t-test p-value = 0.0011) than observed for their common parental strain CBS1483 (Figures 1A, 3A, S2). Although differences in residual maltose concentration were less pronounced and not statically significant (Student's t-test p-value = 0.5844), the evolved strains consistently showed a higher residual maltose concentration (18 to 66 % increase relative to the parental strain). Of the four single-colony isolates, strain IMS0493 showed the best performance, with a 1.18-fold increase

and a 3.7-fold decrease of the residual maltose and maltotriose concentrations, respectively, relative to *S. pastorianus* CBS1483 (Figures 1A and 3).

To test whether strain IMS0493 phenotypically resembled the evolving population from which it had been isolated, its growth and metabolite profile were compared with those of its parental strain CBS1483 in carbon-limited mixed-sugar (SM-Mix) chemostat cultures grown at a dilution rate of 0.05 h^{-1} (Figure 4, Table2). Chemostat cultures of strain IMS0493 rapidly reached a steady state in which extracellular metabolite profiles resembled those observed at the end of the evolution experiment, whilst strain CBS1483 showed the same high residual maltotriose concentrations that were observed at the beginning of the evolution experiments. These results confirmed that, during chemostat-based evolution, strain IMS0493 had acquired an improved affinity for maltotriose which contributed to lower residual sugar concentrations and higher biomass concentrations in steady-state cultures (Table 2) and better attenuation in batch cultures on wort (Figure 3). A faster and more complete conversion of maltotriose by strain IMS0493, relative to it parental strain CSB1483, was also observed in controlled bioreactor batch cultures, grown at 16 ^{0}C on SM-Mix (Figure 3). In these cultures, CO_2 production profiles of the non-evolved strain CBS1483 exhibited two distinct peaks, reflecting diauxic utilisation of first maltose and then maltotriose (Figure S1). In contrast, the CO_2 profile of the evolved isolate IMS0493 showed a single peak (Figure S1) which, as confirmed by analysis of sugar concentrations, reflected co-utilisation of glucose, maltose and maltotriose (Figures 3 and S1).

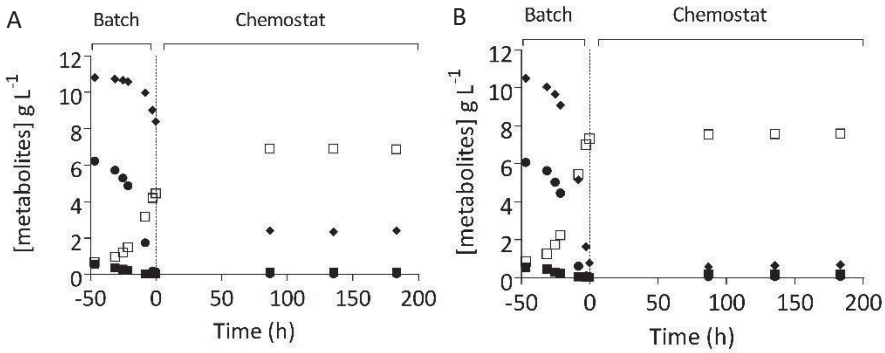

Figure 4: Residual Sugars ((■) glucose, (●) maltose, (♦) maltotriose) and ethanol (□) concentrations in carbon-limited chemostat cultures of *S. pastorianus* strains A CBS1483 and B IMS0493. The parental strain CBS1483 (A) and the evolved strain IMS0493 (B) were grown on SM-Mix (2.5 % glucose, 28.0 % maltose, 42.0 % maltotriose, 26.4 % higher dextrin) at 16 ^{0}C in 1-L working volume anaerobic chemostat cultures at a dilution rate of 0.05 h^{-1}. The vertical line depicts transition from the batch phase (-50 to 0 h) to the continuous-cultivation phase (from 0 h onwards). Sugar concentrations in an independent duplicate experiment differed by less than 2.5 %.

Table 2: Evolved single colony isolate IMS0493 shows improved maltotriose fermentation kinetics compared to its parental strain *S. pastorianus* CBS1483 in a chemostat culture. Biomass yield and rates were determined from steady state measurements of oligosaccharide-limited chemostat cultivations and K_S were calculated from the ^{14}C labelled maltose and maltotriose uptake assays.

	CBS1483	IMS0493
dry weight g L^{-1}	1.23	1.37
$Y_{x/s}$ g g^{-1}	0.08	0.09
$q_{maltotriose}$ mmol g^{-1} h^{-1}	-0.70	-0.76
$q_{maltose}$ mmol g^{-1} h^{-1}	-0.74	-0.66
$q_{ethanol}$ mmol g^{-1} h^{-1}	5.98	6.00
C-recovery (%)	97.87	96.37
Residual [maltotriose] g L^{-1}	2.41	0.68
Residual [maltose] g L^{-1}	0.04	0.06
Final [ethanol] g L^{-1}	6.88	7.60
K_s maltose mM	3.6	3.9
K_s maltotriose mM	7.2	6.5

Improved maltotriose transport capacity

To investigate the role of maltotriose transport kinetics in the improved affinity of the evolved strain IMS0493 for this trisaccharide, sugar-uptake assays were performed with at several [^{14}C-] labelled maltose or maltotriose concentrations. An Eadie-Hofstee fit of the sugar rates revealed that the maximum maltose-uptake rates of the strain IMS0493 and CBS1483 were similar (Figure 5). Substrate-saturation constants (K_m) of strains IMS0493 and CBS1483 were also very similar for maltose (3.9 and 3.6 mM, respectively) but also for maltotriose (6.5 and 7.2 mM, respectively). In contrast, the V_{max} for maltotriose uptake of the evolved strain IMS0493 was over four-fold higher (Student's t-test p-value = 0,03697) than that of the parental strain CBS1483 (23.5 and 4.9 µmol min^{-1} (g dry biomass)$^{-1}$ respectively; Figure 5). The transport capacity for maltose of both strains was approximately 23 µmol min^{-1} ($g_{dry\ biomass}$)$^{-1}$ (Figure 5). These results are consistent with an evolutionary adaptation to sugar-limited chemostat cultivation in which a higher affinity (q_{max}/K_m) for maltotriose primarily resulted from increased expression and/or an improved transport capacity (mol $_{maltotriose}$ (mol $_{transporter}$)$^{-1}$ s^{-1}) of one or more maltotriose transporters in the yeast plasma membrane.

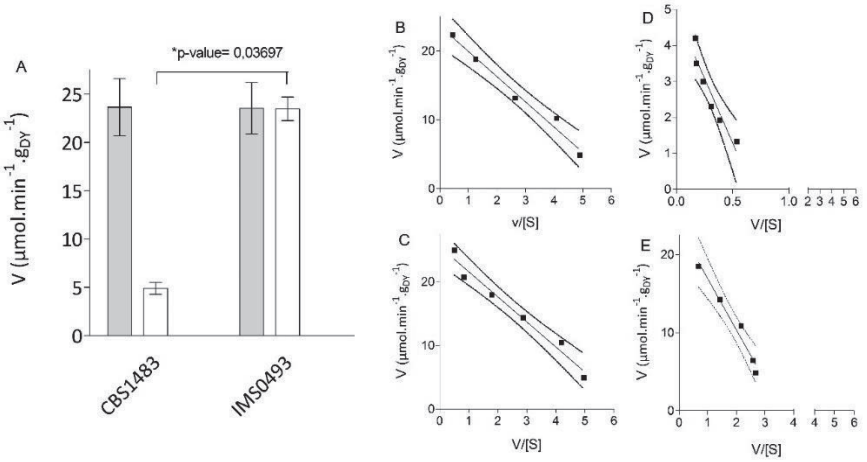

Figure 5: Uptake kinetics of labeled of oligo-saccharides of *S. pastorianus* strains CBS1483 and IMS0493. A- Maximum uptake rates of labeled [^{14}C] maltose (■) and labeled [^{14}C] maltotriose (□) of *S. pastorianus* strains CBS1483 and IMS0493 measured at 20 °C derived from Eadie-Hofstee fits. Values presented are averages ± mean deviation of biological duplicates. * indicates the significant differences between the tested strains with the corresponding Student's t-test p-value. B- Determination of K_m and V_{max} for maltotriose of *S. pastorianus* CBS1483 from Eadie-Hofstee plots [319] with maltose concentration ranged from 2.5 to 25 mM. Intercept with the Y-axis defined V_{max} (23.7 µmol min^{-1} g_{DY}^{-1}) and the slope defined $-K_m$ (-3.6 mM). C- Determination of K_m and V_{max} for maltotriose of *S. pastorianus* IMS0493 from Eadie-Hofstee plots with maltose concentration ranged from 2 to 25 mM. Intercept with the Y-axis defined V_{max} (23.5 µmol min^{-1} g_{DY}^{-1}) and the slope defined $-K_m$ (-3.9 mM). D- Determination of K_m and V_{max} for maltotriose of *S. pastorianus* CBS1483 from Eadie-Hofstee plots with maltotriose concentration ranged from 1 to 25 mM. Intercept with the Y-axis defined V_{max} (4.9 µmol min^{-1} g_{DY}^{-1}) and the slope defined $-K_m$ (-7.2 mM). E- Determination of K_m and V_{max} for maltotriose of *S. pastorianus* IMS493 from Eadie-Hofstee plots with maltotriose concentration ranged from 2.5 to 25 mM. Intercept with the Y-axis defined V_{max} (23.5 µmol min^{-1} g_{DY}^{-1}) and the slope defined $-K_m$ (-6.5 mM). The dash lines represent the 95% confidence interval of the regression line.

Whole-genome resequencing of evolved strain

The alloploid genomes of *S. pastorianus* strains, including the reference strain CBS1483 used in the present study [84], complicate identification of point mutations by short-read resequencing technologies. However, chromosome copy number analysis [267] indicated that, in the evolved strain IMS0493, a two-fold amplification had occurred of a large part of CHRIII, while copy numbers of chromosomes ScI, ScVIII, SeI, SeIX and a segment of the right arm of ScXIV had decreased. The copy number of the affected part of CHRIII had increased from four copies in strain CBS1483 to eight copies in strain IMS0493 (Figure 6A). *S. pastorianus* CBS1483 carries only one version of this chromosome, which is composed of the left arm, centromere and a large part of the right arm of *S. eubayanus* CHRIII and the end of the right arm of *S. cerevisiae* CHRIII [84]. The *MAL2* locus, which is located on the subtelomeric region of the *S. cerevisiae* right arm of CHRIII, seemed not to be affected by the amplification. However, the copy number estimation of individual

maltose transporter encoding gene was disrupted by the inability to accurately assemble paralogous genes (e.g. *SeMALT2* and *SeMALT4* or *MAL31, MAL21, MAL41* and *MAL61*) with short sequencing reads. Thus an attempt to get quantitative estimation of maltose transporter genes was performed by mapping sequencing reads from CBS1483 and IMS0493 on an artificial contig composed of the sequences of all four *S. eubayanus* maltose transporter genes (*SeMALT1, SeMALT2, SeMALT3* and the truncated *SeMALT4* [75]), two *S. cerevisiae MALx1* genes (*MAL11, MAL31,*). To standardize the mapping results, control genes (*SNF5*/YBR289W, *MSH3*/YCR092C, *MES1*/YGR264C, *SeBRN1*/SeYBL097W, *SePDA1*/SeYER178W, *SeATF1*/SeYOR377W, *SeGTR1*/SeYML121W, *SePLC1*/SeYPL268W) located on chromosomes harbouring a *MAL* gene and having no paralogs were selected and together with the highly conserved maltase gene *MAL32* present in all *S. cerevisiae MAL* loci added to the artificial contig. The sequence reads mapping from IMS0493 and its ancestor CBS1483 on these concatenated sequences corroborated the amplification of a large fraction of CHRIII as coverage of *MSH3* was deemed statistically different and the average 1.8-fold higher supporting a doubling of ScCHRIII. All other tested markers did not reveal any significant difference, suggesting that maltose transporter encoding genes were neither gained nor lost in the evolved strain IMS0493 (Figures 6B and S3).

Technology transfer: fermentation performance at pilot scale

Laboratory evolution is a powerful tool to develop strains with improved, innovative traits. Although often performed in academic studies [188, 321-324], these are rarely accompanied by assessment at pilot or full industrial scale. To investigate the industrial relevance of the evolutionary engineering strategy described above, the evolved strain IMS0493 and its parental strain CBS1483 were each tested in duplicate industrial pilot-scale (1000 L) beer-fermentation experiments on a high-gravity 15 °Plato wort (Figure 7). These experiments showed that the acquired phenotypes of the evolved strain were also expressed under industrial conditions. In particular, IMS0493 fermentation showed a significantly lower residual concentration of total fermentable sugars at the end of fermentation (Student's t-test p-value = 0.021944) (Table 3). Total fermentable sugars left in the green beer (green beer is the product after fermentation; it subsequently undergoes conditioning before being bottled) fermented by IMS0493 were 53 % lower than in reference fermentations with its non-evolved parent strain (Table 3). This improvement was predominantly caused by a more complete conversion of maltotriose, whose residual concentration was reduced by 72 % in comparison to residual concentration measured in beer fermented by CBS1483 (Table 3). Although residual maltose concentrations remained below 2 $g.L^{-1}$, they were 83 % higher in experiments with the evolved strain.

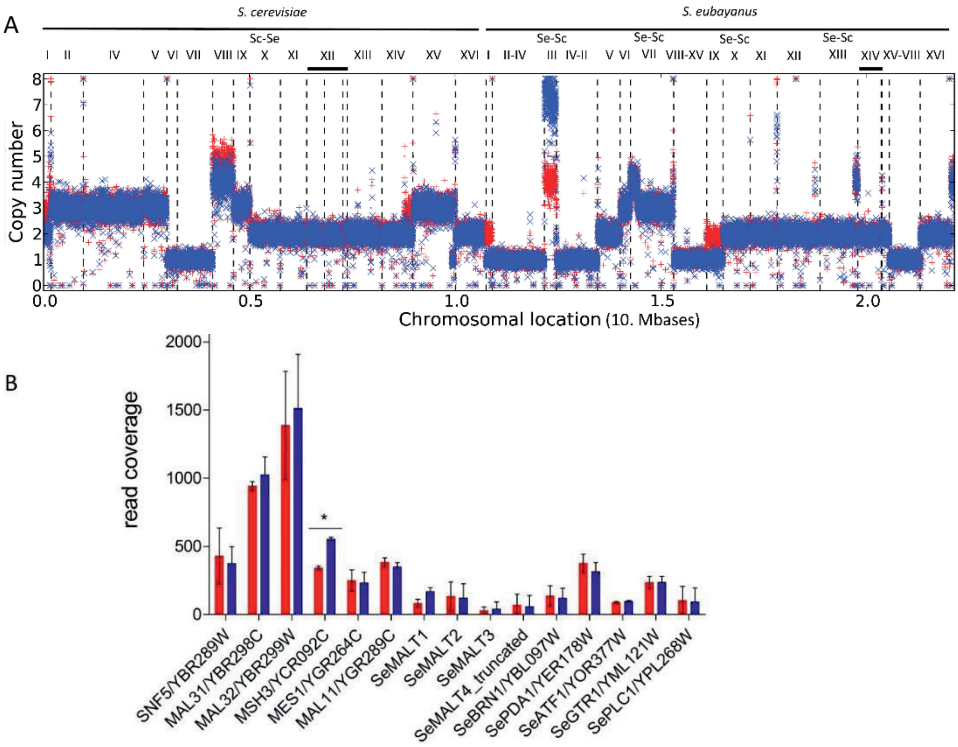

Figure 6: Ploidy differences between the evolved mutant IMS0493 (blue) and the ancestral parent *S. pastorianus* CBS1483 (red). A- The graph represents the ploidy prediction generated with the Magnolya algorithm [93]. Contigs that were de novo assembled by Newbler (454 Life Sciences) and aligned to the reference *S. pastorianus* CBS1483 genome sequence (ASM80546v1) [84] using NUCMER (MUMmer, version 3.21; (www.mummer.sourceforge. net). **B-** CBS1483 (■) and IMS0493 (■) sequencing read mapping on an artificial contig resulting of the concatenation of *S. cerevisiae MAL11, MAL31, MAL32, SNF5, MSH3, MES1* and *S. eubayanus MALT1, MALT2, MALT3, MALT4^{truncated}, SeBRN1*, *SePDA1*, *SeATF1*, *SeGTR1*, *SePLC1* using Burrows-Wheeler Aligner BWA [216]. Coverage was calculated over 100bp-coverage windows. Per gene difference significance of set of 100bp coverage windows from CBS1483 and IMS0493 was statistically assessed using Student's t -test (p-value<0.05). The data represented are averages and standard deviations of per gene 100bp coverage windows. * indicates a difference in coverage between CBS1483 and IMS0493 deemed significant using Student's t-test (*p*-value <0.05).

To explore whether the improved maltotriose fermentation kinetics of evolved strain IMS0493 affected other brewing-related strain characteristics, beers from the pilot fermentations were analysed. Consistent with the analysis on the 1000-L fermentation experiments, the ethanol concentration in beer brewed with strain IMS0493 was 6 % higher than in beer made with the reference strain, while the concentration of residual fermentable sugar was 48 % lower (Student's t-test p-value = 0.043339 and 0.021944 respectively) (Table 3). These results confirmed that the increased fermentation efficiency seen during laboratory experiments could be transferred to an industrial setup [325]. This increase in ethanol concentration would account for a gain of 7.4 % in

volumetric productivity if the green beer were standardized to 5% (v/v) ethanol (Table 3). To investigate whether such standardized beers would comply with lager beer quality standards, aroma compounds were measured (Table 3). Total higher alcohols concentration in standardized bottled beer made with the evolved strain were 9 % higher than in similar beer brewed with *S. pastorianus* CBS1483 but remained within specifications. Additionally, a 40 % lower diacetyl concentration was observed in bottled beer produced with the evolved strain IMS0493 (Table 3). The level measured in IMS0493 (14 µg L^{-1}) was far below the sensory threshold of 20 µg L^{-1}. In comparison the level measured in the non-evolved CBS1483 were just above the threshold (23 µg L^{-1}). Altogether, the aroma profile of beers produced with the two strains met common quality standards for lager beers.

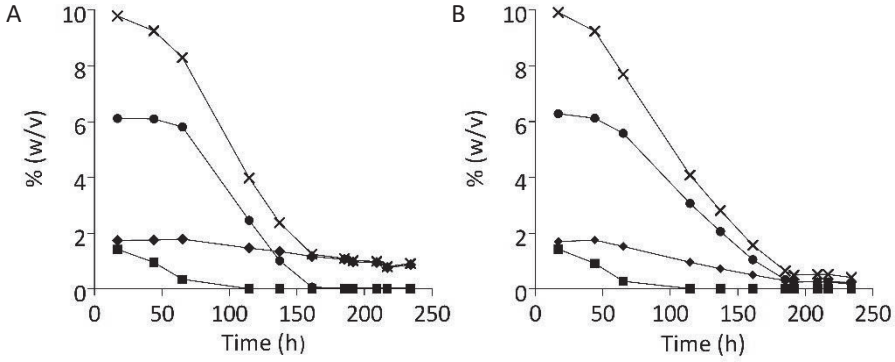

Figure 7: Sugar consumption profiles of *S. pastorianus* strains CBS1483 (A) and IMS0493 (B) in high-gravity wort at 1000-L pilot scale. Total extract (**X**) and Extracellular metabolites ((■) glucose, (●) maltose, (♦) maltotriose) concentrations (expressed % weight/volume) in fermented wort samples were determined by liquid chromatography. Sugar concentrations in an independent duplicate experiment differed by less than 2.5 %.

Table 3: Analysis of green and standardized beers fermented with the evolved isolate IMS0493 and the parental reference CBS1483 in 1000L scale. Higher alcohols, esters and vicinal diketones were determined by gas chromatography and sugars and ethanol were measured by liquid chromatography. Extract data were determined using an alcolyzer beer analyzing system (Anton-Paar). Values presented are averages ± mean deviation of biological duplicates. The *p*-values were calculated using Student's *t*-test. * indicates a difference between CBS1483 and IMS0493 deemed significant using Student's t-test (p-value<0.05).

	unit	**CBS1483**	**IMS0493**	
		Average. ± mean dev	Average. ± mean dev	*p*-value
Green Beer				
Ethanol by volume	%(V/V)	6.32 ± 0.01	6.74 ± 0.03	0.04333*
Total higher alcohols	mg L^{-1}	105.6 ± 0.71	121.9 ± 0.78	0.04107*
Diacetyl (2,3-butanedione)	mg L^{-1}	29.6 ± 8.08	18.9 ± 2.40	0.508841
Total fermentable sugars left	%(m/V)	0.91 ± 0.05	0.47 ± 0.03	0.02194*
Standardized beer				
Ethanol by volume	%(v/v)	5.00	5.00	
pH		4.26 ± 0.01	4.29 ± 0.01	0.062661
Higher alcohols				
Propanol	mg L^{-1}	9.58 ± 0.36	10.84 ± 0.26	0.079166
Isobutanol	mg L^{-1}	12.33 ±0.30	14.70 ± 0.02	0.01552*
Amyl alcohol	mg L^{-1}	61.04 ± 1.43	64.92 ± 0.50	0.081175
Total higher alcohols	mg L^{-1}	82.95 ± 1.37	90.46 ± 0.74	0.04107*
Esters				
Isoamylacetate	mg L^{-1}	3.11 ± .14	3.26 ± 0.07	0.425926
Ethylacetate	mg L^{-1}	23.25 ± 0.67	23.24 ± 0.66	0.581175
Vicinal diketones				
2.3-butanedione (Diacetyl)	µg L^{-1}	23.21 ± 7.54	14.03 ± 1.31	0.498791
2.3-Pentanedione	µg L^{-1}	21.96 ± 8.63	17.71 ± 1.66	0.802657
Others				
Acetaldehyde	mg L^{-1}	2.99 ± 0.43	5.31 ± 0.35	0.175915
Dimethyl sulphide (DMS)	µg L^{-1}	31.42 ± 2.10	48.03± 3.15	0.156564
Sugars				
Glucose	%(w/v)	bdl@	bdl@	NA#
Fructose	%(w/v)	0.02 ± 0.00	0.02 ± 0.00	NA#
Maltose	%(w/v)	bdl@	0.12 ± 0.02	0.052634
Maltotriose	%(w/v)	0.69 ± 0.02	0.20 ± 0.00	0.04237*
Total fermentable sugars	%(w/v)	0.71 ± 0.02	0.35 0.02	0.0219*

@bdl: below detection limit; #NA: Not Applicable.

Discussion

In chemistry and fuel applications, yeast strain development has been intensified and accelerated by novel genome-editing tools such as CRISPR-Cas9 [326]. These techniques enable highly accurate and, when desired, simultaneous gene deletion, nucleotide editing and chromosomal integration of novel genes [170, 206, 327]. Although regulatory frameworks do not preclude use of genome editing for brewer's yeast strain improvement, consumer concerns about use of genetically modified (GM) organisms for food and beverage production discourage industry from implementing this option [174]. In contrast, classical, non-targeted mutagenesis by irradiation (UV, X-ray) or chemical compounds (e.g. ethyl methanesulfonate) continue to be successfully applied in brewer's yeast [325, 328] but requires intensive screening for mutants with beneficial mutations. Additionally, mutagenesis increases the likelihood of secondary mutations, which may negatively affect industrially relevant traits that are difficult to screen in high-throughput set-ups, such as the complex balance of flavour and aroma compounds in the final product.

Evolutionary engineering, is a non-GM technique that is highly suitable for strain improvement in food biotechnology [190]. When a relevant aspect of strain performance can be experimentally linked to growth rate or survival, better performing strains can often be obtained within 50-200 generations, without requiring active mutagenesis [317, 323, 329-331]. In the present study, an *S. pastorianus* strain evolved in laboratory chemostat cultures showed a strongly improved affinity for maltotriose. While the evolutionary strategy explored in this study was designed to improve affinity for maltotriose, costs of pure maltotriose compelled us to use a mixed-sugar substrate, which also contained substantial amounts of maltose. This choice likely contributed to the acquisition of a maltose/maltotriose co-consumption phenotype acquired by the evolved *S. pastorianus* strain IMS0493, which enabled a faster wort fermentation (Figures 3, 7, S2). Accelerated fermentation probably contributed to a faster reduction of diacetyl and, thereby, to the observed lower concentrations of this off-flavour in beer brewed with the evolved strain. The aneuploid, alloploid genomes of lager brewing yeasts are diverse and dynamic with respect to chromosomal copy number variations [94]. Indeed, whole-genome sequencing revealed several copy number variations in strain IMS0493, which evolved for improved maltotriose affinity. The genome dynamics of industrial lager brewing yeast might also be associated with decreased diacetyl formation in IMS0493. Acetolactate is converted to diacetyl under the action of acetolactate synthase, an enzyme comprising two subunits. The catalytic subunit is encoded by *ILV2* and the regulatory subunit encoded by *ILV6* a gene located on CHRIII. Although the *ILV6* copy number has been associated with variation in diacetyl production in *S. pastorianus*, a reduction in copy number was associated with lower diacetyl [332] while overexpression of either of the *ILV6* alleles led to increased diacetyl formation [81]. These results would disagree with the results of this study which would associate a doubling of *SeILV6* copy number with a

reduction of diacetyl. This would suggest that other genetic elements might influence the level of this undesired metabolite. Extensive chromosome copy number variations observed in IMS0493 did not negatively affect flavour profile in pilot-scale fermentations. The properties acquired by the evolved strain enabled an increased production of beer from the same amount of wort, with a reduced maltotriose content and a flavour profile that was fully compatible with lager brewing specifications. This result demonstrates the potential of evolutionary engineering to improve specific traits of brewing yeasts without disturbing other industrially relevant phenotypes.

Sugar-transport studies indicated that the improved affinity of evolved strain IMS0493 was caused by a three-fold higher maltotriose transport capacity than that of the parental strain. However, the genetics of maltose and maltotriose transport in *S. pastorianus* have not yet been fully resolved by functional analysis of individual oligosaccharide transporters. This is especially relevant for a novel *SeAGT1* allele identified in *S. pastorianus* [83, 134, 145] but that so far could not be identified in available *S. eubayanus* sequences [34, 75].

Identification of causative mutations remains the next challenge. Whole-genome sequencing, using Illumina short-read technology, which allows for accurate analysis of chromosomal copy number variations [267], did not reveal changes in the copy number of known or putative sugar transporter genes in the evolved strain *S. pastorianus* IMS0493. The genetic basis for improved maltotriose uptake in this strain is therefore more complicated than a simple amplification of a known transporter gene. In haploid or homozygous yeast genomes, single-nucleotide mutations in non-repetitive sequences can be easily identified by short-read sequencing technologies [323, 333, 334]. However, in alloploid brewing yeast genomes, their identification remains a critical challenge, despite fast developments in sequencing technology [313]. While tools such as GATK [265] selectively search for heterozygous positions in diploid genomes, no such tools are currently available to identify heterozygous single-nucleotide mutations in aneuploid genomes. In the case of *S. pastorianus*, such an identification is especially challenging because the *S. cerevisiae* and *S. eubayanus* subgenomes are closely related and, locally, share near-complete sequence identity [34, 74, 75]. Furthermore, recent studies have established that *S. pastorianus* strains can be highly aneuploid, with individual chromosome copy numbers ranging from one to five in a single genome [77, 78, 82-84]. Consequently, phasing of two or more variable positions in a single gene is only feasible if they are captured in a single sequencing read. Similar to the problems encountered in this study, genome-wide expression analysis could not identify the exact molecular basis for improved performance of mutagenized *S. pastorianus* selected for growth in very high gravity wort [328] and at high osmolarity [331]. Research in this area is likely to profit from fast developments in long-read technology (e.g. Pacific Biosystems and Oxford Nanopore), which will facilitate reconstruction of single alleles and haplotype chromosome versions and, thereby, development of new bioinformatics solutions to identify relevant mutants in hybrid, highly aneuploid genomes.

Acknowledgements and funding

This project is funded by the Seventh Framework Programme of the European Union in the frame of the SP3 people support for training and career development of researchers (Marie Curie), Networks for Initial Training (PITN-GA-2013 ITN-2013-606795) YeastCell. Brian Gibson was additionally supported by grant 276480 from the Academy of Finland. We thank Virve Vidgren for technical support with the transport assays.

Chapter 5: Outlook

Lager beer, which is brewed with hybrid *S. pastorianus* yeast strains, makes up a large fraction of the global beer market. After a period in which this market was dominated by a small number of large brands, there is now a growing customer demand for product variety. This trend towards diversification is illustrated by the rapidly increasing abundance and popularity of craft breweries. On this background, now and in the future, fast and reliable development of novel brewing yeast strains is required to improve the economics, sustainability and product quality of large-scale industrial brewing processes as well as to increase product diversity. This strain development can be based on random mutagenesis and/or selection, on evolutionary or genetic engineering of existing production strains and/or on constructing new hybrid yeast strains.

As our understanding of brewing yeast genomes and of the physiology of brewing yeasts and their industrially relevant traits increases, new metabolic engineering approaches offer interesting technical opportunities for rapid and high precision strain improvement. An example is the use of CRISPR-Cas9, or similar tools, for targeted modification of yeast genomes (in some cases even without introduction of foreign DNA). Recent, adventurous experiments have demonstrated how metabolic engineering of yeasts can enable the production of hop-derived compounds in beer. By introducing heterologous plant genes encoding linalool and geraniol synthases and by simultaneously increasing precursor supply, beer with elevated monoterpene concentrations and thus a hoppy flavour could be produced [335]. In the described study, not only favour, but also sustainability of the brewing process was addressed since the cultivation of hops uses high quantities of energy, water, and fertilizers. Brewing yeast strains could principally be genetically engineered to be more resistant against stress factors in the brewing environment, to make industrial fermentations more efficient as well as to develop novel products. However, in view of recent developments in GMO regulation, especially in Europe, it is unlikely that targeted genetic modification, even when limited to 'self-cloning' with CRISPR-Cas9-based genome editing, will be implemented for large-scale brewing within the next decade. In the current climate with respect to discussions on GMO applications, the risks of negative impacts for especially large brewers, related to lack of consumer acceptance, do not outweigh potential benefits in terms of production costs and product diversity. The great potential of the advancements in genetic engineering could potentially be of use especially in the agricultural sector, for producing energy efficient, high-yield crops, which is indispensable in ensuring food supply for a growing world population as well as with regard to climate change. On this background it is of great importance that scientific developments go along with an effective communication and education towards both the general public and political stakeholders.

The current impasse in the introduction of targeted genetic modification in industrial food and beverage fermentation processes, increases the urgency to develop efficient, 'non-GMO' methods for brewing yeast strain improvement. This thesis illustrates the potential of two of such approaches: evolutionary engineering and generation of novel hybrids of industrial *Saccharomyces* strains. The generation and use of novel hybrid strains is demonstrated by the model study described in **Chapter 3** of this thesis. In this study, an interspecies hybrid was constructed that combined and even improved features of both parent strains, namely fermentative capacity and cryotolerance. Hybridization offers a fast and simple way to combine relevant properties of different natural and/or mutagenized strains, thus further extending the diversity and improving the performance of brewing strains. When required, strain development methods based on hybridization can be combined with non-targeted mutagenesis to further increase genetic diversity. Such a strategy was applied by Diderich et al. who generated Pof$^-$ *S. eubayanus* variants (not producing phenolic off-flavour) via UV mutagenesis, which were subsequently crossed with likewise Pof$^-$ *S. cerevisiae* strains resulting hybrids with phenotypic characteristics of common *S. pastorianus* lager brewing yeasts [118]. Newly created hybrid yeast strains have a great potential in satisfying the customers' demand for diversity in beer styles and can also be applied in wine and citer as well as for bioethanol production.

Once more with regard to strain improvement resulting 'non-GMO' strains, the applicability of adaptive laboratory evolution for the improvement of complex traits in industrial strains was demonstrated in this thesis. This strategy is especially useful for improving strains with complex, alloploid genomes such as the *S. pastorianus* strain studied in **Chapter 4**. Here, maltotriose uptake by lager yeasts, which often presents a bottleneck in industrial fermentations, was improved by means of evolutionary engineering in continuous carbon-limited cultures. An additional example for improving complex traits is the enhanced resistance to osmotic and ethanol stress in high-gravity media brewing yeasts acquired after adaptive laboratory evolution [168]. Those studies illustrate the potential of evolutionary engineering strategies for improving the efficiency of industrial fermentations.

The research described in the thesis also shows how whole-genome-sequencing techniques, which become ever more precise and cost-effective, can be used for analysis of brewing yeast genomes. In combination with high-throughput analytical platforms for analysing brewing-related aspects of strain performance (e.g. flavour profiles), it will become increasingly more realistic to efficiently isolate predefined genotypic variants with desired phenotypes from random mutagenesis and hybridization techniques. Given the diversity and complexity of the genomes of brewing yeasts, advanced bioinformatics, including machine learning approaches, will be essential to develop such new platforms.

Analysis of genome sequences will further play a major role in understanding oligosaccharide metabolism. In order to facilitate physiological and regulatory analysis of the four potential maltose transporters in the mentioned strain in **Chapter 2**, the

complete sequence of all corresponding genes was obtained from a, by the use of Oxford Nanopore MinION sequencing technology, newly generated genome assembly. Sequencing technology based on long reads presents a valuable possibility to study especially genes and gene families that are located in subtelomeric regions. Those regions are evolutionary hotspots, often including repetitive elements and duplications, and contain genes involved in nutrient uptake and stress tolerance. Using long-read sequencing techniques, the incredible evolutionary flexibility and variability of subtelomeric genes will further be illuminated in the near future and the genetic accessibility of subtelomeric regions will be greatly improved. In addition to variations in subtelomeric regions, chromosome copy number variations (CCNVs) are a key contributor to industrial relevant traits such as stress tolerance fermentative capacity, diacetyl production, and flocculation and are found in industrial strains of plenty applications [336]. CCNV occurs extensively in lager brewing hybrids and was shown to be a result of prolonged laboratory evolution as shown in **Chapter 4**. Breakpoints of chromosomal rearrangements in *S. pastorianus* strains can be used to trace back whether lager brewing hybrids are derived from one or several hybridization events [79]. In which way exactly CCNV has benefitted *S. pastorianus* hybrids during their evolution has yet to be studied.

In order to explain the early evolutionary events that had led to the rise and predominance of *S. pastorianus* lager brewing yeasts, in **Chapter 3**, an interspecies *S. cerevisiae* x *S. eubayanus* hybrid was constructed and characterized. The results of this study elucidate that the tested features (cryotolerance and oligosaccharide utilization) have been key selective advantages for lager brewing hybrids in the harsh, man-made environments of lager beer production. In order to develop a deeper understanding of the evolutionary history of *S. pastorianus* and its maltose and maltotriose transporters, it will further be significant to look into a large set of potential parental *S. cerevisiae* and *S. eubayanus* strains. On this background, studying the sequence of *MAL* gene families of *S. eubayanus* strains of different lineages but also of lager brewing strains themselves, will be of high importance.

The fermentation of maltose and maltotriose by brewing yeasts is the major determinant for the efficiency of the production process and is important for the quality of the final product. To better understand the contribution of both parental genomes of *S. pastorianus* to maltose transport, in **Chapter 2**, *MAL* transporter genes of the *S. eubayanus* type strain CBS12357 were analysed. Two out of four present *MALTx* genes were shown to be the main responsible maltose transporters in *S. eubayanus* CBS12357 even though all four *MALTx* genes, overexpressed in a maltose-transport-deficient strain background, were confirmed to encode functional proteins. This discrepancy is explained by a possible insufficiency in activator binding sites and underlines the necessity to study the wholesomeness of transport and hydrolysis and regulation of both in order to fully understand oligosaccharide metabolism in brewing-related yeast strains.

Chapter 4 of this thesis illustrates how results from a laboratory-scale evolution experiment could be directly translated to pilot-scale brewing. Pilot brewing fermentation with the evolved yeast strain showed comparable results to the laboratory characterizations and beer produced from those fermentations exhibited flavour profiles satisfying the requirements for lager beers. Pilot fermentations are expensive and, by definition, low throughput. To increase throughput of strain improvement programmes, scalability of laboratory experiments (at most mL scale) to at least pilot scale (at least m^3 scale) will be of paramount importance. Given the complex medium composition and process dynamics of industrial brewing processes, reproducing industrially relevant beer fermentation conditions in high-throughput screens represents a challenge, that will require inputs from process engineers as well as from microbiologists.

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Acknowledgements

This thesis is the result of a slightly over four year long lasting educational as well as personal adventure and it would not have been possible without the support and individual involvement of many people I met during this journey. I like to take the opportunity to express my gratitude to all them here.

First, I like to thank my supervisors Jack and Jean-Marc. You have been a substantial support throughout my whole PhD. You cautiously guided me or sometimes pushed me when I needed it and you were always eager to discuss any upcoming questions. I have learned so much, scientifically and personally, from both of you and am very grateful that I had the chance to work with you. Thanks a lot, you are outstanding examples as scientists for me and many other young researchers who are at the very begin of their career.

To Pilar, Marijke, Erik and Matthijs: You have been of great help with many things in the lab and assisted me in planning and executing experiments and were always eager to discuss and solve any question. Thanks to Marcel for your help in analyzing and interpreting sequencing data. I owe quite a bit to the involvement of all of you. I am also thankful for the help in preparing experiments that Apilena, Astrid, and Jannie provided. Your contribution to my and everyone else's work at IMB is very valuable and should never been underestimated. Thank you very much.

A special thanks goes to the whole IMB group. I am not going to list names because on one hand the group expanded quite a bit within those four years so that I can barely keep track and on the other hand, and most importantly, every single one at IMB made a contribution to the friendly, welcoming atmosphere I encountered here. Thank you so much for stimulating scientific discussions as well as for fun times outside the lab. I could not have wished for another group to perform my PhD in. So many smart and motivated people on one spot, that's really something special.

During my PhD I had the opportunity to supervise many BSc and MSc students. To Carina, Josien, Joan, Julie, and Lieke: Thanks a lot for your contribution to my work. I have learned from all of you and I hope you could also take out something useful from time we worked together.

I consider it a great privilege to have been a PhD candidate and researcher within the Marie Curie ITN YEASTCELL. Thanks to John and Francesca to organize and keep together this amazing international network of young researchers and experienced PIs which gave me the opportunity to get to know colleagues from universities, industry and research institutes from all over Europe and make friends within the yeast community. I learned a lot on yearly summer schools and meetings at conferences which is a bonus that a PhD candidate outside such a network would not automatically get. Also, thanks to the Seventh Framework Programme of the European Union and the SP3 people support for

training and career development of researchers: No research would be going on without funding of course.

Thanks to HEINEKEN for ensuring me a salary in the final year of my PhD and thanks to Jan-Maarten and Niels for giving me the opportunity to make my research into an actual product. Your support and trust were of great value for me. The time in the lab and pilot facility at HEINEKEN was a very valuable experience. Similar to this, I also had the opportunity to perform part of my research at VTT. Thanks to Brian and his group: I felt very welcome and immediately part of the team. That made winter in Finland less depressing than I had initially thought. I learned a lot during both those research exchanges and am very grateful to have gotten those opportunities. Thanks to everyone involved.

I further thank Marit and Irina for the contribution to **Chapter 3** of this thesis. It was a great opportunity for me to get involved in the work with interspecies hybrids and a perfect starting point for my research on brewing yeasts.

A special thanks goes to Pilar and Nick. I am extremely happy you agreed to be my paranymphs and support me during the final phase of my PhD as you also have been of great help during my time at IMB, both scientifically and personally. Thank you both so much.

Last but not least I want to express my gratitude to the people who have been important for me outside of work which then again was crucial as backing up this beginning of my scientific career. Thanks to my parents for always encouraging and supporting me with every step of my educational journey from high school until my PhD. You are always positive and understanding and what is maybe even more important: You raised me in a way that stimulated my curiosity for anything that is going on in my surrounding. By teaching me how much we can learn from nature and how important it is to take good care of it, I think, I acquired quite some crucial values for a life as a scientist and for life in general. Thanks as well to my brother and to my friends in Germany and in the rest of the world: It was always helpful to be around you guys occasionally when PhD life got too crazy. I found it extremely refreshing to have the main topic of conversation switch from science to other important things in life once in a while. A special thanks also goes to Christiaan: You are an enrichment for and give stability to my life. Thanks for always being there for me, for getting me grounded when I am stressing out and overthinking everything and for supporting me in all I do. You believing in me and my abilities means a lot to me and really gives me confidence. Thank you for having my back.

Curriculum vitae

Anja Brickwedde was born on October 13, 1988 in Nordenham, Germany. After finishing high school in 2008 in Osnabrück, Germany, she enrolled in the BSc program Environmental and Industrial Biology (ISTAB) at the University of Applied Sciences in Bremen, Germany in 2008. During her studies, she did a placement at TNO (Netherlands Organization for Applied Scientific Research) in Zeist, the Netherlands in 2010 where she worked on fungal strain development (*Aspergillus niger*) with An Li and Peter Punt. Also, during her Bachelor studies, in 2011, Anja enrolled in a Ecotoxicology minor at the University of Gothenburg in Sweden, where she studied for one semester. Anja's Bachelor thesis was performed as part of a virtual company at the University of Applied Sciences in Bremen under the supervision of Tilman Achstetter and Gerd Klöck and was focussed on setting up a quality management system for the production of yeast extract as source of glutamate. After her Bachelor studies, Anja enrolled in the MSc program Environmental and Industrial Biology (ISTAB) at the same school in Bremen, Germany in 2012. Here she specialized in the track industrial biology. For her Master end project, she conducted research on optimizing itaconic acid production by *Aspergillus niger* through metabolic engineering and controlled batch fermentation at TNO in Zeist, the Netherlands under the supervision of An Li, Karin Overkamp and Peter Punt. During her studies in Bremen, Anja was actively involved in setting up a life science student initiative (btS e. V.) and in organizing events for students.

After graduating in 2013, Anja started her PhD project in 2014 within the Industrial Microbiology group at Delft University of Technology, the Netherlands under the supervision of Jean-Marc Daran and Jack Pronk. Main objectives of the research were studying maltose and maltotriose metabolism in brewing related *Saccharomyces* yeasts as well as improving the industrial performance of a *Saccharomyces pastorianus* lager brewing hybrid by means of adaptive laboratory evolution. The project was funded by the Seventh Framework Programme of the European Union in the frame of the SP3 people support for training and career development of researchers (Marie Curie), Networks for Initial Training (PITN-GA-2013 ITN-2013-606795) YeastCell (https://yeastcell.eu/). At the partner institutions VTT Technical Research Center of Finland Ltd, Espoo and HEINEKEN Supply Chain, Global Innovation and Research, Zoeterwoude, the Netherlands, where Anja performed 3 months of her research each, she worked under the supervision of Brian Gibson and of Jan-Maarten Geertman and Niels Kuijpers, respectively. The outcomes of Anja's PhD research are described in this thesis.

List of publications

1. Hebly M, Brickwedde A, Bolat I, Driessen MR, de Hulster EA, van den Broek M, Pronk JT, Geertman JM, Daran JM, Daran-Lapujade P: ***S. cerevisiae* x *S. eubayanus* interspecific hybrid, the best of both worlds and beyond**. FEMS Yeast Research 2015, 15:fov005. doi: 10.1093/femsyr/fov005.

2. Brickwedde A, van den Broek M, Geertman J-MA, Magalhães F, Kuijpers NGA, Gibson B, Pronk JT, Daran J-MG: **Evolutionary Engineering in Chemostat Cultures for Improved Maltotriose Fermentation Kinetics in *Saccharomyces pastorianus* Lager Brewing Yeast**. Frontiers in Microbiology 2017, 8:1690. doi: 10.3389/fmicb.2017.01690.

3. Salazar AN, Gorter de Vries AR, van den Broek M, Wijsman M, de la Torre Cortés P, Brickwedde A, Brouwers N, Daran J-MG, Abeel T: **Nanopore sequencing enables near-complete de novo assembly of *Saccharomyces cerevisiae* reference strain CEN.PK113-7D**. FEMS Yeast Research 2017, 17:fox074. doi: 10.1093/femsyr/fox074.

4. Brickwedde A, Brouwers N, van den Broek M, Gallego Murillo JS, Fraiture JL, Pronk JT, Daran J-MG: **Structural, Physiological and Regulatory Analysis of Maltose Transporter Genes in *Saccharomyces eubayanus* CBS 12357^{T}**. Frontiers in Microbiology 2018, 9:1786. doi: 10.3389/fmicb.2018.01786.

5. Hossain AH, Li A, Brickwedde A, Wilms L, Caspers M, Overkamp K, Punt PJ: **Rewiring a secondary metabolite pathway towards itaconic acid production in *Aspergillus niger***. Microbial Cell Factories 2016, 15:130. doi: 10.1186/s12934-016-0527-2.

6. Li A, Pfelzer N, Zuijderwijk R, Brickwedde A, van Zeijl C, Punt P: **Reduced by-product formation and modified oxygen availability improve itaconic acid production in *Aspergillus niger***. Applied Microbiology and Biotechnology 2013, 97:3901-3911. doi: 10.1007/s00253-012-4684-x.

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