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Use of non-*Saccharomyces* yeast for beer fermentation as illustrated by *Torulaspora delbrueckii*

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“Inside all of us is Hope.

Inside all of us is Fear.

Inside all of us is Adventure.

Inside all of us is… A Wild Thing.”

- Maurice Sendak, Where the Wild Things Are

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Preface and peer reviewed publications

1. Michel M., Meier Dörnberg T., Jacob F., Methner F., Wagner R., Hutzler M. (2016). **"Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications."** Journal of the Institute of Brewing & Distilling 122: 569-587 DOI: 10.1002/jib.381

2. Michel M., Kopecká J., Meier-Dörnberg T., Zarnkow M., Jacob F., Hutzler M. (2016). "**Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model.**" Yeast 33 (4): 129-144. DOI: 10.1002/yea.3146

3. Michel M., Meier-Dörnberg T., Schneiderbanger H.,Haselbeck K., Zarnkow M., Jacob F., Hutzler M. (2017). "**Optimization of beer fermentation with a novel brewing strain *Torulaspora delbrueckii* using response surface methodology**" Technical quarterly MBAA 54 (1): 23-33 DOI: 10.1094/TQ-54-1-0215-01

4. Michel M., Meier-Dörnberg T., Kleucker A., Jacob F., Hutzler M. (2016). "**A new approach for detecting spoilage yeast in pure bottom-fermenting and pure *Torulaspora delbrueckii* pitching yeast, propagation yeast, and finished beer**." Journal of the American Society of Brewing Chemists 74 (3): 200-205 DOI: 10.1094/ASBCJ- 2016- 3148-01

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Eidesstattliche Erklärung

Hiermit versichere ich eidesstattlich, dass ich die vorliegende Arbeit selbstständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen entnommen sind, wurden als solche kenntlich gemacht.

Die Arbeit wurde in keiner gleichen oder ähnlichen Form einer anderen Prüfungsbehörde vorgelegt.

Freising,

Notations

% v/v	Volume percent
a or α	Mating type
ADP	Adenosine diphosphate
AGT	Maltose plasma membrane transport protein
ATP	Adenosine triphosphate
CCD	Central Composition Design
CO_2	Carbone dioxide
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
e.g.	For example
EMS	Ethyl methanesulfonate
et al.	et alia (and others)
GAI	Extracellular glycosylated glucoamylases
GAL	Hexose plasma membrane transport proteins
GMO	Genetically modified organisms
GRAS	Generally Recognized As Safe
hl	Hectoliter
HXT	Hexose plasma membrane transport proteins
IBU	International bittering units
KAR 1	Gene for cytoplasmic trait
MAL	Maltose plasma membrane transport protein
MDR	Multidrug response
MNNG	Methylnitronitrosoguanidine
Mphx	Maltose plasma membrane transport protein
Mtt	Maltose plasma membrane transport protein

NAD^+	Nicotinamide adenine dinucleotide
NGS	Next generation sequencing
No.	Number
p	Significance level
PCR	Polymerase chain reaction
PDRE	Pleiotropic drug-response element
P_i	Phosphate
POF	Phenolic off-flavor
RAPD	Random amplified polymorphic DNA
RGT	Hexose plasma membrane transport proteins
RSB-PCR	Repetitive sequence-based PCR
RSM	Response surface methodology
S.	*Saccharomyces*
S288c	First complete sequences *S. cerevisiae* strain
SD	Standard deviation
SNF	Hexose plasma membrane transport proteins
STA	Extracellular glycosylated glucoamylases
T.	*Torulaspora*
T9	Strain abbreviation for *Torulaspora delbrueckii* T9
V-ATPases	Plasma membrane proton transport enzyme

Summary

The applied brewing yeast strain contributes decisively to the aroma profile, taste, smell and mouth feel of the produced beer. The mass production of beer has led to a selection of a small number of high-performing *Saccharomyces* yeast strains, making it relatively easy to produce beer on a large industrial scale. However, as yeast has an immense impact on beer flavor and attributes, the selection of this small number of yeast strains has left behind the aromatic richness of beers.

In recent years a movement called craft brewing has been growing. This type of brewing uses special malts and special hops to increase the aromatic richness of beer. Since yeast is one of the main flavoring agents for beer, a variation of this mandatory ingredient can further enrich the beer's aroma. Besides the specially selected brewing strains, there are many partly uncharacterized genera of yeast in addition to *Saccharomyces,* each with many species and strains, giving a wealth of possibilities for potential beer fermentation. Many of these non-*Saccharomyces* yeasts are known to brewers as contaminants that cause major changes in the aromatic profile of beer. Not all of these sensorial changes, however, are considered bad, as beers like Berliner Weiße and Lambic show which aromatic richness relies on the interaction and impact of differing yeast genera.

A method to predict the capability of a non-S*accharomyces* yeast strain with regard to beer fermentation as well as its potential use were investigated in this dissertation.

One of the most time-consuming steps in searching for new yeast strains for brewing is phenotypical characterization. In this scientific work a phenotypic characterization protocol (screening) was developed to predict the performance of a yeast strain in beer fermentation. Firstly, literature was consulted to sum up pre-existing protocols and trials with non-*Saccharomyces* yeast for beer fermentations. As a result, saccharide and amino acid utilization in all malt barley wort, hop compound and ethanol tolerance as well as flavor forming were chosen as the main phenotypic challenges. After the successful compilation, execution and evaluation of a screening protocol with ten strains of the *Torulaspora delbrueckii* species, a promising strain was found as the new brewing strain *T. delbrueckii* T9 and was taken a step further in the characterization program. The beer fermentation performance of this particular strain was optimized using response surface methodology, varying fermentation temperature (15-25°C) and pitching rate (50-120 x10^6 cells/mL). Before this, the strains behavior in propagation and dissolved oxygen demand in wort was investigated.

The combination of a 20 °C fermentation temperature and a pitching rate of 60 x10^6 cells/mL as well as a wort oxygenation of 10 mg/L dissolved oxygen was found to be sufficient. In

contrast to brewing yeasts used previously, propagation showed very high cell concentrations after 28 hours of 400 x10^6 cells/mL at the highest vitality and viability. The beer was judged to be very fruity with strong notes of blackcurrant. Furthermore, a temperature-dependent change in flavor could be observed. At a fermentation temperature of 15 °C the beer had a strong honey-like flavor, changing to blackcurrant at 20 °C and to red wine-like at 25 °C. To ensure the quality of the finished product and the pureness of the pitching yeast, a method was successfully developed to detect cross contaminations of top-fermenting spoilage and brewing yeast in one to five days without prior incubation. A micro fermenter with a pressure detector was therefore incubated with pure and spiked samples of differing brewing and spoilage strains. Spoilage yeast contaminations of 0.001 % in 1 x 10^6 cells/mL pitching yeast could be detected within an average of 5 days.

Zusammenfassung

Die, bei der Bierherstellung, verwendete Hefe beeinflusst das Aromaprofil, den Geschmack, den Geruch und das Mundgefühl eines Bieres maßgeblich. Im Zuge der Massenproduktion von Bier wurden, aus einer ehemals großen Vielfalt, einige wenige Hochleistungshefen der Gattung *Saccharomyces* selektiert, die die industrialisierte Bierfermentation möglich machten, die Aromenvielfalt des Bieres hingegen stark einschränken.

Unter dem, aus den USA übernommenen, Synonym Craftbeer werden Spezialmalze, sowie neue Hopfenzüchtungen in der Bierproduktion verwendet, um wieder neue Aromen in das Bier einzubringen. Da die Hefe einen der größten Einflüsse auf das Aroma des Bieres hat, ist eine Variation des Hefestammes eine weitere Möglichkeit die Aromenvielfalt zu erweitern. Neben der Gattung *Saccharomyces* existieren viele, teils noch nicht beschriebene Hefegattungen. Einige davon kommen als Kontaminationen in der Brauerei vor und können eine starke sensorische Veränderung der Biere zur Folge haben. Dass diese sensorische Veränderung nicht negativ sein muss, zeigen Spezialbiere wie z.B. die Berliner Weiße oder das Lambic, deren Aromenvielfalt auf dem Einsatz verschiedener Hefespezies beruht.

Eine Methode zur Einschätzung der Anwendbarkeit in der Bierherstellung von nicht-*Saccharomyces* Hefen in Reinkultur, sowie die mögliche Anwendung in der Brauerei soll in dieser Arbeit untersucht werden.

Die zeitaufwändige Charakterisierung stellt, unabhängig von dem gewählten Anwendungsgebiet, eines der Probleme für die Erschließung neuer Hefen dar. In der hier vorliegenden Arbeit wurde ein phänotypisches Charakterisierungsprotokoll entwickelt, welches zu einer schnellen Einschätzung der Fähigkeit isolierter, natürlich vorkommender Hefen auf ihre Anwendung in der Bierfermentation befähigt. Hierfür wurde zunächst eine ausgiebige Literaturrecherche durchgeführt, die bereits verwendete, sowie angewandte Tests und Gärversuche mit verschiedenen nicht-*Saccharomyces* Hefen zusammenfast. Als wichtigste Eigenschaften wurden die Verwertung wichtiger Kohlenhydrate und Aminosäuren aus der Bierwürze, die Hopfen-- und Ethanol-Toleranz, sowie die Aromastoffbildung beschrieben.

Nach erfolgreicher Zusammenstellung, Durchführung und Evaluierung des phänotypischen Charakterisierungsprotokolls mit 10 Stämmen der Spezies *Torulaspora delbrueckii*, konnte ein Stamm als potentielle Brauhefe identifiziert werden. Er verstoffwechselte alle wichtigen Würzezucker, konnte in der Anwesenheit von Hopfensäuren wachsen, zeigte eine Toleranz gegenüber 5 % Ethanol und bildete fruchtige beerenartige Aromen. Im weiteren Verlauf wurde der Fermentationsprozess dieses *T. delbrueckii* Stammes T9 mit der Variation

verschiedener Fermentationsparametern auf die Herstellung eines Bieres mit durchschnittlichem Alkoholgehalt von 5 % v/v optimiert. Für diesen Schritt wurden die optimale Gärtemperatur zwischen 15-25°C, sowie Anstellzellzahl zwischen 50-120 x10^6 Zellen/mL, das Verhalten in der Propagation und der Einfluss von Sauerstoff in der Anstellwürze untersucht. Für die Variation der Fermentationstemperatur und der Anstellzellzahl wurde eine Response-Surface Methode angewendet. Ein optimales Ergebnis erbrachte die Kombination von 20 °C Gärtemperatur und 60 x10^6 Zellen/mL bei einem gelösten Sauerstoffgehalt der Anstellwürze von 10 mg/L. In der Propagation zeigten sich im Vergleich zu normaler Brauhefe sehr hohe Zellzahlen (bis 400 x10^6 Zellen/mL) sowie eine sehr gute Viabilität und Vitalität nach 28 Stunden. Das Hauptaroma des fertigen Bieres wurde von den Verkostern als sehr fruchtig mit starkem Geschmack nach schwarzer Johannisbeere beschrieben. Es konnte weiterhin eine Veränderung des Aromas der Biere bei steigender Fermentationstemperatur beobachtet werden. So änderte sich das Aroma bei 15 °C von Honig Noten über starkes Johannisbeerenaroma bei 20 °C zu einem starken Rotweinaroma bei 25 °C. Im weiteren Verlauf der Arbeit wurde eine neue Qualitätssicherungsmethode basierend auf Gasbildung entwickelt. Diese ermöglicht den Einsatz des neuen Hefestammes in der Brauerei sowie die Detektion von möglichen Kreuzkontaminationen mit *Saccharomyces cerevisiae* in ein bis fünf Tagen ohne Vorinkubation.

1 Introduction and motivation

Discussions of brewer's yeast today refer to some highly domesticated [1], fast and predictively fermenting *Saccharomyces cerevisiae* as well as *Saccharomyces pastorianus* strains [2–5]. These yeast strains, especially *S. pastorianus* strains, ferment brewer's wort into beer efficiently and economically in a short period of time. The overall aroma and taste of beer is largely shaped by the fermenting yeast used in the process [6–10]. It converts the fermentable carbon and nitrogen sources present in wort into the main fermentation products ethanol and carbon dioxide. Furthermore, strain-specific volatile and non-volatile compounds which are described as secondary metabolites, and contribute highly to flavor, are produced during fermentation [8–11]. As only a couple of strains are used by the big brewing companies today this overall aroma impression does not vary greatly for the big mass produced [12, 13].

The overall single reason for using some major brewing strains is the biological and economical benefits [14]. Since fermentation is one of the most time- and space-consuming steps in the beer production process it has always been a field in need of innovation. To save space, the volumes of the fermentation vessels were increased by using high cylindroconical fermentation vessels [15, 16]. To reduce time, high-gravity brewing was invented, which increased the yield of the fermentation, saved energy, cleaning, and effluent costs [17–19]. The yeast strains used for these fermentations had to be specially selected as the stress coming from a high fermentation vessel with high gravity wort negatively affects yeast performance [13, 20]. Consequently, a few big companies that produce most of the beers with these yeast species polarized the world beer market, restricting the aroma and flavor variety [12, 14]. The majority of beer produced today is of the lager variety, which is produced by the bottom-fermenting yeast *S. pastorianus* [14]. *S. pastorianus* ferments efficiently at low temperatures, produces a clean aroma profile and has a high level of various stress resistances, which makes it very useful for mass producing beer [6, 13, 14, 21, 22].

A trend that can be observed over the last decade is a growing interest in craft-produced beer due to the aforementioned uniformity and insipidity of the majority of the products offered by the big brewing companies [23]. As consumers become more aware of how variable beer can be, the demand for these products increases [24]. New innovative and keenly experimental breweries are launching all over the world, reviving old beer styles and creating new beers [12, 23–25]. The hop industry has adapted to the new demand and has increased its variety of special hops for new flavors. As yeast is one of the main aromas and flavor-shaping agents in beer production, demand for new yeast strains is increasing. Yeast strains

for new beer styles are therefore sought by brewers and scientists in many different ways [23, 25, 26]. One method is to use old yeast strains that were kept in storage, though these are not as high performing. These yeasts produce differing flavors and aromas to what is commonly used at present. Another way predicted by Steward in 1986 was the widespread use of novel brewing strains that were genetically modified [27]. Since then, scientists have taken a variety of approaches to genetically modify yeast strains [2, 28]. However, the opposition shown by public opinion means that they have not yet found their way into breweries [13]. Another promising alternative is the search for new brewing strains in nature as there are potentially many still undiscovered varieties [29–32]. The industrially used yeast strains, especially in the brewing sector, only cover a small number of the virtually unlimited number of yeasts found in the environment [29], some of which might be useful to brewers. Non-conventional (*i.e.*, non-*Saccharomyces*) yeast have been successfully applied to improve flavor and aroma in mixed and pure fermentations for wine, cacao and other fermented beverages [33–42]. These yeasts and many others can be key to discovering novel aromas and flavors in beer [23, 25, 31, 33, 36, 43–45]. To be able to find new brewing strains, the nature of beer fermentation using *Saccharomyces* brewing strains has to be taken in account and adapted to the new yeast strains selected from nature [3, 4, 23, 25, 26].

The thesis publications are therefore organized in four parts:

1. Review of applied non-*Saccharomyces* yeast species in beer fermentations with a detailed description of practical applications and produced secondary metabolites.
2. Development of a screening method for non-*Saccharomyces* yeast to predict the ability of the strains to ferment beer wort with a verification of the results by the application of ten *Torulaspora delbrueckii* strains.
3. Establishment of an optimization protocol to implement a non-*Saccharomyces* brewing strain of the species *Torulaspora delbrueckii* in the brewery by optimizing wort aeration, propagation and fermentation.
4. Development of a novel methodology to ensure purity and quality of beers produced by the species *Torulaspora delbrueckii* and bottom-fermenting yeast.

1.1 History of beer yeast

The tradition of producing beer, bread and wine can be traced back thousands of years to prehistoric times [3, 5, 28]. The driving force behind fermentation was not known to the early brewers, bakers and wine makers besides the fact that sugar-containing foods and liquids spontaneously fermented when left alone for some time. As the air that these foods were exposed to as well as some of the ingredients, contained a variety of yeast and bacteria, the outcome of these fermentations was not predictable. It was inefficient and the taste was

probably not always desirable [46]. The first documented steps towards some kind of a predictable beer fermentation were taken by Sumerians approx. 6,000 years ago by inoculating non-fermented food with a small fraction of pre-fermented food to start a new fermentation [5, 46, 47].

Brewing technology has been promoted and the production of beer increased ever since. As a result of the variety of anti-bacterial properties it contained, beer was one of the safest beverages to consume in times before the invention of water treatment [48]. It had a low pH (around 4.2), which harmed the growth of toxic gram-negative bacteria, a certain concentration of ethanol, hop acids and carbon dioxide, which made it a harsh environment for any bacteria to live [49]. There are reports by Sambrook of medieval times where rich households would consume 750-1500 hl of beer *per annum*. A servant at that time was allowed to have one gallon (respectively 3.8 liters) a day [50]. Up to today, beer is one of the most consumed fermented beverages in many countries. In 2015 an average of 105.9 liters was consumed by the German population, which equals 0.29 liters per day [51].

Intensive research into yeast, however, did not start until the end of the 17th century due to a lack of knowledge and technology. In 1680, Antoni van Leeuwenhoek found small round shapes in fermenting liquid by looking though a very simple microscope, but was not able to show that they were linked to fermentation [52]. In 1789 Antoine van Lavoisier described the nature of fermentation as a chemical change in the fermentation of wine, which was not linked to any microorganism [53]. In 1837 and 1838 the research on beer supported by the improvement of microscopes reached a high point with Schwann and Cagniard-Latour who found living yeast to be linked to fermentation [54–56]. Cagniard-Latour was able to measure the size of yeast cells and described them as small globules with a diameter of 6–9 µm [55]. Pasteur then used this knowledge twenty years later to identify yeast as the fermentation agent and showed that wild yeast and bacteria, if present in the fermentation, would spoil wine and beer. He also reported that aerobic and anaerobic microorganisms and yeast have a much higher demand for sugars when in an anaerobic environment [56, 57]. In 1842 bottom-fermenting yeast, which had only been used by Bavarian brewers until that time, was brought to the country of Czechoslovakia. From there it was taken to Denmark and at almost the same time introduced to American breweries in Pennsylvania [27]. The idea of a pure fermentation was then implemented by Emil Christian Hansen in 1883 in Denmark at the Carlsberg brewery [58]. When focusing his research on yeast he was able to cultivate four different pure strains of bottom-fermenting yeast, of which he found one to be suitable for beer fermentation. That strain was called `*Carlsberg Yeast no. 1'* [58]. Due to this name, bottom-fermenting yeast was hereinafter named *Saccharomyces carlsbergensis* [27].

In 1886, Hansen developed a propagation system in the Carlsberg brewery together with Jansen to be able to supply the brewery with adequate pure culture yeast. Since that time the use of pure cultures of brewing yeast became common practice for brewers producing bottom-fermenting beer [27]. However the ale brewers, mostly from the United Kingdom where ale was most common, did not immediately adopt this technique. Their product diversity at that time partly relied on mixed cultures of ale yeast containing mainly two to three but also up to five strains at once [59]. Furthermore, no adaptation was found for special beers, which are still partly spontaneously fermented today e.g. Lambic, Geuze or Berliner Weissbier. The nature of most of these fermentations is still not completely discovered as many different microorganisms are involved in varying concentrations and time spans [12, 60–62].

Since that time scientists have been trying to speed up and increase the yield of the fermentation process. In 1930, a large step was taken towards mass production as cylindroconical fermentation vessels were invented [16]. Typical sizes for large brewery fermentation vessels today are 1500-2000 hl with a height of 10-20 m. Due to the height, the pressure at the lowest point of the fermenter can reach up to 2 bar which can result in over carbonation and harm yeast growth [6]. However, the cone makes it easy to crop the yeast from the bottom as it is collected in the cone when it flocculates after cooling [16]. Making it easy to crop bottom-fermenting yeast in closed vessels might also have been a small advantage for lager production as the yeast could be cropped in a sterile way and reused, saving money, space and time in the brewery [27].

In 1935 another big discovery in yeast research was made by Winge, who discovered that cells of *Saccharomyces* were diploid and could be produced by combining two haploid spores [63]. Having that knowledge, he discovered one of the first methods to intentionally create a new brewer's yeast that had previously been used for plants and animals [64]. He had the idea that breeding was possible, and used two haploid spores of different parental yeast strains to form a new diploid yeast strain with characteristics of both parents [28, 64]. From that time on yeast research was expanded to include genetics and molecular biology [27, 28]. There is still one issue with this idea today. Yeast strains coming out of the laboratory are diploid and it is comparatively easy to change their genetics. However, industrially used strains are mostly alloploid or polyploid, which partly ensures genetic stability in the brewing process but also makes it much harder to modify them [28, 65, 66].

Brewing scientists today have the advantage that *S. cerevisiae* has been used for fundamental research in cell biology and genetics [65]. The *S. cerevisiae* strain (S288c) was the first eukaryote for which a fully characterized genome sequence was available as a result of the collaboration of many scientists [67]. The high interest in *S. cerevisiae* by many other industrial

branches such as biotechnology provides a further advantage to our knowledgebase as a large number of researchers work on improving and finding new strains and also on the genetics of other yeast species [68]. Today, researchers all over the world use next generation sequencing to try to map the genes responsible for phenotypes, which might be interesting to industry of any kind [65, 68]. However, the public opinion on genetic modification as well as the legal position of the use of these microorganisms is still not very positive [13, 28].

Recently, some groups of scientists have started to compare industrially used *Saccharomyces* strains by their whole genome sequence [1, 5, 14, 69]. These yeast strains cluster when compared by wine, beer and other fermentation industries but also show some strains that are used in one industry but belong to a different industrial sector [1, 5, 69]. These clusters also show traits of domestication as a result of years of usage in a man-made environment, producing in the industrially used strains of today [1]. However, the results also show that some of the strains used today carry traits of other genera and are sometimes interspecies hybrids [69].

In summary, yeast research has improved the fermentation of wort into beer by highly domesticated *Saccharomyces* yeast strains to virtual perfection [70]. This progress was possible due to the developments in the technology, genomics, proteomics and metabolomics of yeast as well as selection and domestication over centuries [5, 13, 71]. There are still some gaps but in the overall scheme of things, knowledge about fermentation has advanced considerably and the overall quality and efficiency of breweries has reached a high level. In all these positive impacts and optimization processes however, the product itself was limited in its sensorial complexity [7, 12, 23, 72]. All the knowledge gathered in past decades can be used by researchers to discover new brewing yeast strains, which might enrich the sensorial complexity once again [7, 13, 25, 26, 32].

1.2 Obtaining new brewing yeast strains

To acquire new brewing strains, two main requirements have to be taken into account. The method to find or create new yeast strains has to be implemented and the field of use has to be determined. The following paragraphs will deal with the different methods of finding new yeast strains followed by a description of the methods developed here.

There are different techniques that can be used to either explore or create new yeast strains for industrial e.g. brewing purposes. They can be divided into four groups of methods, which can be summed up as using natural yeast biodiversity, artificial selection, direct evolution or genetic modification [26]. Common to all of these techniques to date is the fact that the phenotype (a special characteristic that a strain or a species can have e.g. morphology, physiological or biochemical properties) has to be investigated for the specific field of use. This

investigation is necessary before it is possible to tell if the desired phenotype is present for the discovered or created yeast strain. To date there is a lack of knowledge on the full interaction between phenotype and genotype, which makes fast phenotypic screening important. With increasing knowledge and better technologies e.g. next generation sequencing (NGS) this interaction has been investigated in recent years by numerous groups of scientists but has not yet been fully discovered [73–75].

1.2.1 Natural yeast biodiversity

The first and of course, the oldest technique is to use natural yeast biodiversity. As mentioned in the above section 1.1 it is known that humans made use of its natural diversity thousands of years ago [3, 5]. The "main workhorse", as *Saccharomyces cerevisiae* is often referred to, in fermentation is one of approx. 1500 yeast species that have been characterized for different fields so far. However, this number is just an infinitesimal part of what natural biodiversity has to offer [29, 71]. Even in the *Saccharomyces* genus, natural diversity is unbelievably extensive. Scientists have reported that the degree of genetic diversity of a spatially separated wild *Saccharomyces cerevisiae* population on a small island in southern China is comparable to the genetic diversity of the complete human population [71]. As this represents only one species where thousands of different species with different strains are also found in the same environment, this indicates how large the diversity in yeast can be. That is why screening for a certain phenotype from naturally occurring yeast has become a common tool for finding new strains [26]. Different scientist teams have started screening yeast strains of differing species out of big collections for different industrial purposes for many years [26, 76–78]. However, very little has been done to find new brewing yeasts apart from *Saccharomyces*. A promising approach of finding new strains that will perform in a similar way, is to screen yeast strains that are related to the environment of beer fermentation or that occur in the beer fermentation as spoilage yeast. As these yeast strains might already be adapted to the environment, they might also be able to ferment or utilize beer wort in a similar way [28, 30]. Proof of this theory has been given by various scientists that found indigenous wild yeast to be promising starter cultures for wine [79, 80] or to be replacements for bakers yeasts that were used in Brazilian biofuel production [81]. It should be noted that some yeast strains can produce toxins. Before searching for specific characteristics of interest in food fermentation the GRAS (Generally Recognized As Safe) database should be consulted for the specific species [82].

1.2.2 Artificial selection

This approach covers methods to increase the pre-existing yeast diversity using techniques that generate genetic diversity from a single strain or by shuffling genomes of multiple strains. However, the emerging strains are described as non-genetically modified yeast (in some regions strains produced by protoplast fusion are considered to be GMO (genetically modified organisms)) and can therefore be used in any industrial fermentation [28]. These man-induced changes in the genome can be performed by mutagenesis [83], sexual hybridization [84], asexual hybridization [85] or evolutionary engineering.

Mutagenesis describes the creation of mutants induced by physical or chemical mutagens. Examples of physical mutagens are ultraviolet rays or ionizing radiation. Frequently used chemical mutagens are EMS (ethylmethanesulfonate) or MNNG (methylnitronitrosoguanidine) [83]. The physical or chemical mutagens force a mutation of the genome (e.g. change of nucleotides, transversions, point mutations and cluster mutations) [86], which result in various mutants some of which can have a desired phenotype that has to be selected by phenotype investigation [26, 86].

Sexual hybridization, also called mating, has been common practice in agriculture to produce hybrids. These hybrids can be produced from two parents from different subspecies but the same species (intraspecific), from two different species but the same genus (interspecific) or from two different genera (intergeneric) [87]. Yeast hybridization covers some methods (direct mating, rare mating, mass mating and genome shuffling) that are used to create new hybrids from haploid spores of diploid yeast cells [87]. The main procedure will be described on the process of direct mating. Firstly, the yeast strains with differing desired phenotypes are forced to form spores by placing them on a nutrient-insufficient medium e.g. acetate medium [84]. These spores, which either have the mating type a or α (comparable to human genders male and female), harbor one set of chromosomes of the mother cell (haploid). If an a and an α mating type are put together by a micromanipulator they form a new cell, which harbors a double set of chromosomes (diploid). This can result in new combinations of genes, which might support a more desired phenotype such as cryotolerance, ethanol tolerance or higher aroma production [84, 88]. Hybridization, however, has some disadvantages. Most industrially used strains are polyploid, have low sporulation viability or do not sporulate at all. The produced hybrids can have an unstable genotype and therefore change after a certain number of fermentations, losing their desired ability in the fermentation [89]. Therefore, they have to be genetically stabilized by means of repeated fermentations and stability testing using fingerprinting [89].

Asexual hybridization covers the methods of protoplast fusion and cytoduction. Protoplast fusion describes a procedure where the cell walls of differing yeast cells (same species or differing species) are enzymatically removed, resulting in protoplasts (cells without cell walls). These protoplasts are fused, generating a new cell with a fused nucleus of both cells and therefore the characteristics of both cells. The new cell is able to grow and reestablish a cell wall, enabling it to multiply again. This method is used for yeasts that do not sporulate or are polyploid, making them unable to mate [85]. In this approach, the resulting strain contains both chromosomes of the parental strains. If only the cytoplasm (containing different cytoplasmic factors e.g. mitochondria) of one parental strain but both chromosomes (nucleus) of the other parental strain are meant to be in one new cell, cytoduction is performed. Here, the *KAR 1* gene of the parental strain containing the targeted cytoplasmic trait is deleted. Then the protoplasts are fused as described above resulting in a cell with the nucleus of one parent and the cytoplasm of both parental strains [90].

1.2.3 Direct evolution

Direct evolution has also been described as adaptive or experimental evolution [91]. It covers methods of adapting a population of yeast cells (or any other microorganism) to an environment. The environment is chosen according to the desired phenotype, e.g. for fermentation, high sugar concentration, low temperatures, high ethanol concentration. Cells that grow faster or ferment stronger because of a spontaneous mutation due to the environment are selected, continuously repitched and selected again [92]. As these cells have an advantage towards the rest of the population, these cells will succeed in the fermentation and enrich over generations, producing a high cell number of fast-fermenting mutants. A simple example is the serial repitching of brewing yeast, which can result in a higher-performing population (also in a lower-performing population in case of petite mutants [93]), after a couple of fermentation in contrast to the first pitches [94].

1.2.4 Genetic modification

This field covers methods that directly manipulate a yeast's genome using biotechnological tools. As the genome is directly modified, the resulting organisms need to be labeled as GMO and are subject to GMO legislation. The pharmaceutical industry has been taking advantage of GMO for many years to produce human proteins with yeast or bacteria for therapeutic treatments [95]. However, direct use in food production is prohibited by law by most European countries and their future use is still controversial [28]. The field of genetic modification covers many complex methods, the basic principle of which will be explained briefly in the following section.

Manually produced DNA or foreign DNA from other microorganisms can be inserted into the genome of the yeast cell, changing its phenotype e.g. fermentation ability, resistances, flavor forming and many other attributes. Genes that have been unintentionally produced can be removed or changed by mutation, giving countless variation options. Two major and efficient ways of inserting foreign DNA into yeast cells have been described [96]. The first one is to use so-called plasmids where a plasmid vector is introduced into a host yeast cell [97]. This vector transports a certain DNA fragment, which will be integrated in the host's genome. It can carry the information to produce a certain protein and also the information for a biochemical pathway. Following integration, this information is given to all descendants of this particular cell [98]. The production of specific compounds requires the integration of multiple differing plasmids to change the genome for the desired purpose, and this decreases the genetic stability [99]. The second technique is the so-called fixed integration. Here, a gene is replaced by a manipulated gene one by one. This action has the benefit that the gene given to the decedents will be as stable as the original. As the yeast genome is relatively small this technique is very practical [100]. Most modified genes are responsible for gene expression or regulation, giving the opportunity to increase the production of a desired compound. A balance of these gene functions, however, is very important as high gene expression does not necessarily mean a high production of a compound [101].

The greater understanding of the genotype-phenotype interaction as well as an increase in the whole genome sequence data now makes it partly possible to link the phenotypes to the genotype. As most of the desired phenotypes for industrial purposes are quantitative (controlled by multiple genetic loci), this has led to approaches such as quantitative trait loci mapping. These approaches will make it possible to screen any yeast using its DNA for any desired phenotype, to change the gene, and predict performance [68].

1.3 Applicability of yeast in beer fermentation

When searching for new brewing strains, the purpose of the new yeast in the application of the fermentation of beer should be defined [2, 13]. There are multiple applications on how a yeast strain can be integrated into the fermentation of beer, which results in different products:

1. It could be used as a pure culture to completely ferment wort (approx. 70-85 % final attenuation) and produce a "usual gravity beer" with a wort containing approx. 12 °P original gravity. Another possibility could be the fermentation of a high gravity wort with about 13-22 °P such as *S. pastorianus* or *S. cerevisiae* [13, 102, 103].
2. It could be used to partly ferment the wort and produce an alcohol-free or low alcohol beer such as *Saccharomycodis ludwigii* [104–106].

3. It could be used in a pre- or mixed fermentation with pre-existing brewing strains, providing additional benefits such as a highly desired aroma or flavor as suggested by various authors [25, 26, 72].
4. It could be used as a post-fermentation agent, changing the flavor, acidity and carbon composition of the beer such as *Brettanomyces bruxellensis* in lambic beers [7].

In this particular work, the author chose the first application, to search for new brewing yeast strains in the non-*Saccharomyces* sector, which will ferment all hopped barley malt wort to produce a respectable beer of average alcohol content. The following will therefore focus on this specific field of use.

1.4 Phenotypic challenges for new brewing yeast

Whichever method is chosen to find the new strains, the phenotype has to be investigated to predict whether the applied yeast strain will ferment hopped wort into a respectable beer. The phenotypically challenging properties are:

- the ability to grow in the presence of hops, as some hop compounds have antiseptic properties which can influence yeast growth [107].
- the fermentation of saccharides present in all malt barley wort to predict the fermentation ability of the yeast strain [103]. In particular, the utilization of maltose and maltotriose as the main wort saccharide is mandatory [108, 109].
- the tolerance towards ethanol as a normal gravity beer fermentation will lead to about 5 v/v% of alcohol [12]. Ethanol can inhibit fermentation due to toxicity [110, 111].

The influence of these phenotypic properties of *Saccharomyces* brewing yeast will be described in the following paragraphs. As most of these phenotypic investigations have not yet been performed for non-*Saccharomyces* yeast, a summary of the available literature will be given.

1.4.1 Influence of hop-originating substances on yeast

The mandatory addition of hops to boiling wort has a long-standing tradition in beer production [112, 113]. It adds different positive influences to the beer regarding taste, physicochemical stability and microbial stability [107, 113, 114]. Hops (*Humulus lupulus* Linnaeus) is a member of the Cannabaceae family. The *Humulus* genus includes further *H. japonicas* and *H. yunnanensis* but only *H. lupulus* is used for beer production. The hop cones or parts of them are added to the wort as whole cones, pellets or extracts. The hop cones include many different substances – bitter acids, hop oils and polyphenols are the most important ones for brewers [113]. In terms of bitter acids, this relates to humulone and

lupulone homologs. The large fraction of hop oils (containing several hundreds of substances) were classified by Sharpe and Laws in 1982 into three groups of hydrocarbons, oxygenated compounds and sulfur-containing compounds [115]. About 50-80 % of total hop oils are hydrocarbons, in particular monoterpenes, which mainly contribute to hop flavor [116]. About 3 % to 6 % of the hops' dry weight is polyphenols. They have a positive impact as an antioxidant in beer and contribute to foam stability [117].

The main antiseptic properties come from humulone homologs (α-acids), lupulone homologs (ß-acids) and isomer products *(cis/trans-iso-α*-acids) [118, 119]. While boiling the wort with hops, α-acids (humulone, cohumulone and adhumulone) are isomerized into *cis*- and *trans-iso-α*-acids depending on the duration of boiling and amount of hops as well as the *α*-acid content of the added hops [120, 121]. There are three more homologs of humulone, post-, pre- and adprehumulone but their quantity of total *α*-acid content is very low in comparison with humulone (35-70 %), cohumulone (20-55 %) and adhumulone (10-15 %) [122]. The average concentration of *iso-α*-acids in lager beer amounts to 20–30 mg/L [107, 119]. The amount of *iso-α*-acids can vary due to the beer type from 5 to over 100 mg/L [112, 121]. As *α*-acids are isomerized, the actual remaining amount in beer reaches 1-25 mg/L. The amount of ß-acids (co-, post-, ad-, prelupulone and lupulone) for lager beer was reported to be between 0-2 mg/L whereas the amount in highly hopped craft beers is still not described in literature [118, 119, 121]. All these acids have been reported to harm the growth of gram-positive but not gram-negative bacteria [114]. However, there has so far been very little research into the influence of these compounds on yeast specific to brewing [111].

Saccharomyces cerevisiae and *S. pastorianus* have been found to be highly tolerant against bitter acids. Only concentrations of *iso-α*-acids much higher than present in beer had inhibitory effects on their growth [123]. In 2010 Hazelwood *et al.* investigated the influence of hop acids on the growth of *Saccharomyces* yeast and different mutants to investigate the influence of hop acid tolerance on eukaryotic cells [107]. A reference liquid containing only sugars was fermented as well as a spiked liquid containing 0.2 g/L and 0.5 g/L of *iso-α*-acids. In an analysis of the genome-wide transcriptional response they found 120 genes up-regulated and 198 genes down-regulated when comparing the reference with the spiked sample. When looking at the function of the up-regulated genes, they found that most of them were responsible for stress response, detoxification and iron ion transport. They reported three major mechanisms that could be responsible for *iso-α*-acid tolerance in these yeasts (Figure 1). Firstly, a modification of the cell wall was reported, which decreased the access of *iso-α*-acid into the cell. Secondly, MDR (multidrug response) transporters belonging to the PDRE regulon act (pleiotropic drug-response element) move *iso-α*-acid to the external medium. Thirdly, *V-ATPases* acidified the vacuoles, resulting in a comparable low pH value inside the

vacuole and an import of *iso*-*α*-acids. Inside the vacuole, chelate complexes are formed with zinc or iron. These complexes could not exit the vacuoles and were stored. The influence of *iso*-*α*-acids on the growth of the yeast strain used was described as moderate [107]. No investigation into the hop acid tolerance of other species besides *Saccharomyces* has been reported by other authors [111, 113]. As tolerances vary between genera and species for many different antiseptic agents [124], the influence on growth and therefore fermentation behavior of new brewing yeasts should be investigated when screening for brewing ability.

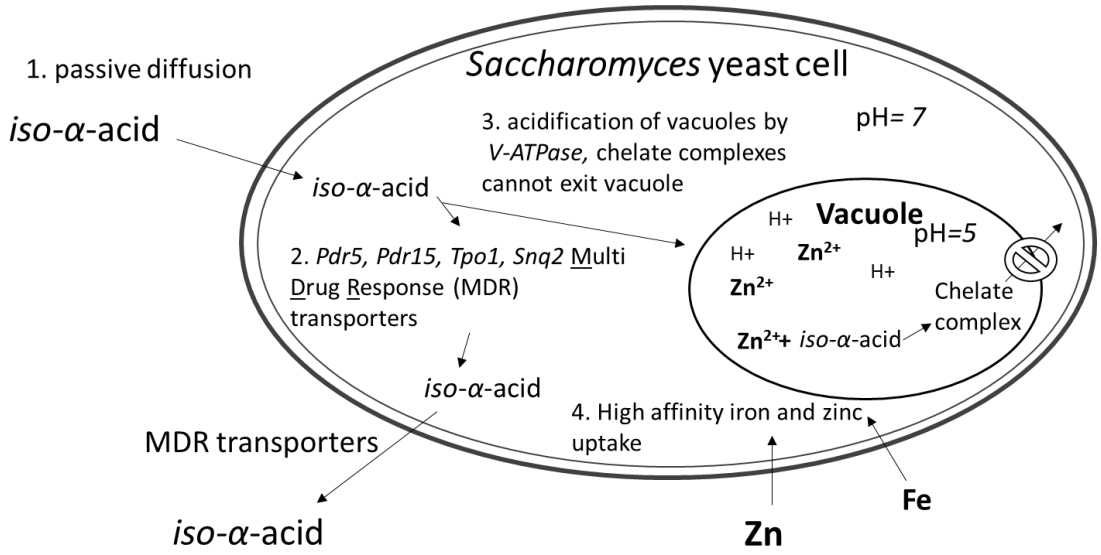

Figure 1 Potential iso-α-acid resistance mechanisms of *Saccharomyces* brewing yeast according to Hazelwood *et. al* [107]

1.4.2 Transport and fermentation of wort saccharides

Saccharides present in a standard gravity (approx. 12 °P) all barley malt wort are glucose (10-15 %), fructose (1-2 %), sucrose (1-2 %), maltose (50-60 %), maltotriose (15-20 %) and differing dextrins (20-30 %) [103, 125].

To be able to metabolize these saccharides, yeast has to be able to transport them into the cell. For saccharide utilization the transport itself determines the amount and speed much more than the intracellular enzyme breakdown [126]. Yeast cells shield themselves from the surrounding medium by a cell wall, a plasma membrane as well as a periplasmic space in between. Most saccharides can freely pass though the cell wall as it is a porous layer consisting of linked glucan and mannan. However, they cannot pass the plasma membrane. This requires the action of transport proteins [127]. *Saccharomyces* brewing yeast strains have different transport mechanisms to pass saccharides through their plasma membrane. Depending on the saccharide, it is taken up intact by transport proteins, meaning it is not broken down before being transported across the plasma membrane [108] (Figure 2). The monosaccharides

glucose, fructose, the disaccharides maltose and the oligosaccharide maltotriose are taken up intact. Sucrose is broken down before transport. The enzyme invertase, which is excreted by yeast inside the periplasmic space, breaks down sucrose into fructose and glucose, which can then be taken up by transporters [128].

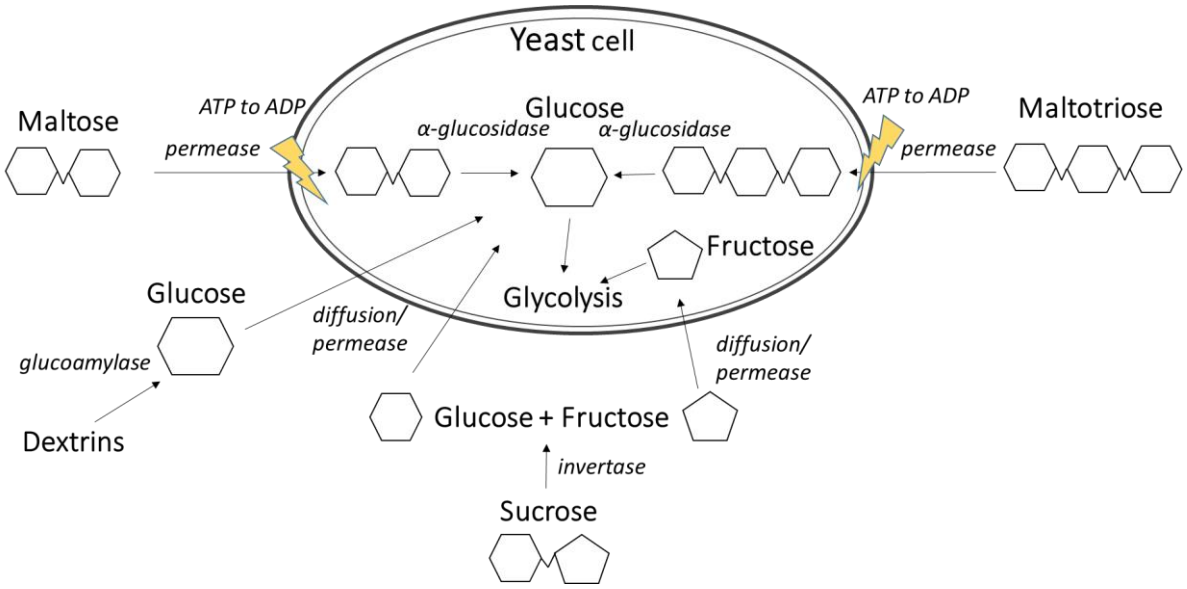

Figure 2 Wort saccharide transport into yeast cell (modified according to Steward [103, 129])

For most yeast species the transport of the monosaccharides (hexoses), glucose and fructose is performed passively by specific hexose permeases using facilitated diffusion, meaning that no energy is required for the transport (Figure 2). *Saccharomyces cerevisiae* has about 20 hexose transport proteins. These proteins are named *HXT1* to *HXT17*, *GAL2*, *SNF3* and *RGT2 [130]*. The differences between the hexose transporters in different yeast species has yet to be investigated. For *Kluyveromyces lactis, Schizosaccharomyces pombe* and *Pichia stipites* just a few differing transport proteins have been reported [130]. Most of these transporters work along a gradient at moderate extracellular hexose concentrations [127].

The transport of maltose and maltotriose by *Saccharomyces* brewing yeast is performed by almost all the same transport proteins, having a higher efficiency for maltose than maltotriose [131]. As mentioned above, maltose and maltotriose are the major saccharides present in all malt wort with more than 50 % of the total saccharide concentration [103, 125]. The ability to transport and ferment these two saccharides is therefore mandatory for brewing yeast to produce a complete fermented beer. However, a difference has been reported for *S. cerevisiae* and *S. pastorianus* in the uptake and complete consumption of maltotriose [132]. Energy is required to transport these two saccharides as the mechanism is based on proton symport. For each saccharide, one proton is co-transported inside the cell. This proton is transported outside the cell by an ATPase ion pump using the energy of one ATP molecule

hydrolysis to ADP and P_i [103]. These transport proteins are called *MALx1* (*x* stands for loci *1-4* and *6*), *AGT1*, *Mphx* and *Mtt1*. *Mal31* and *Mal61* transport maltose but not maltotriose [108]. The transport of these two saccharides in *Saccharomyces* brewing yeast is further linked to the concentration of glucose. Glucose causes a catabolite repression and inhibition, which delays the uptake of maltose and maltotriose until about 60 % of glucose has been utilized [133].

Dextrins are not utilized by *Saccharomyces* brewing strains. However, some species such as *Saccharomyces cerevisiae* var. *diastaticus*, *Brettanomyces* and *Saccharomycopsis* have a system of three unlinked genes that belong to the glucoamylase multigene family [134–136]. These genes (*STA1*, *STA2* and *STA3*) encode three extracellular glycosylated glucoamylases GAI, GAII, and GAIII, which can break down dextrins into glucose, which can then be taken up by glucose transporters [136].

Some yeast strains harbor the genetic information in their DNA to transport and utilize differing carbon sources [131, 137, 138]. However, most of the time a functional regulator, transporter or parts of the genes are missing. Which results in a phenotype that does not show utilization of these specific carbons [137, 139].

Some non-*Saccharomyces* yeast strains have been reported to utilize maltose [135, 136, 140, 141]. As maltotriose is mostly only important for brewers, bakers and distillers, very little research has been conducted on this carbohydrate for yeast other than brewing yeast [141–143].

After transporting the saccharide into the cell, the yeast has to be able to ferment it into ethanol. Whether a yeast strain can transport and ferment all wort carbohydrates depends on its genetic complement and therefore enzymatic endowment [103]. Differences in the saccharide metabolism in differing species arise for the mechanisms of uptake, differing isoenzymes and regulation of fermentation and respiration. The actual central carbon metabolism, the Embden-Myerhoff glycolytic pathway, is very homogenous in all of them [144]. Glucose and fructose are directly converted into pyruvate by the Embden-Myerhoff glycolytic pathway. Maltose and maltotriose are broken down by the enzyme maltase into 2 or 3 glucose molecules respectively before entering the same pathway [133].

To form ethanol, yeast must be able to ferment pyruvate. Fermentation of pyruvate usually takes place whenever the electron transport chain is unusable after glycolysis. This happens when there is no final electron receptor, oxygen (anaerobe). To generate ATP, a CO_2 molecule is enzymatically cleaved (decarboxylation) from pyruvate, resulting in a molecule of acetaldehyde. Acetaldehyde is further reduced into ethanol by alcohol dehydrogenase, regenerating one NAD^+ (Nicotinamide adenine dinucleotide) [12]. By fermenting glucose,

yeast gains 2 mol of ATP versus a gain of 38 mol ATP by respiration. It has been reported that only half of all yeast species are able to ferment saccharides and produce ethanol and CO_2 [145]. The ability of *Saccharomyces* brewing strains to ferment all wort saccharides efficiently might have come from the domestication process, a selection performed by humans as some researchers believe [5, 30]. They found that >90 % of all investigated *Saccharomyces* brewing strains were able to utilize and ferment all of these saccharides while less than 20 % of the investigated wild undomesticated *Saccharomyces* strains had this ability [30].

The fermentation ability for all the main wort saccharides is important phenotypic information which needs to be investigated for a potential new brewing yeast [146].

1.4.3 Ethanol tolerance

Ethanol is one of the main fermentation products produced along with carbon dioxide by fermenting yeast species. Even though yeast produces ethanol, it is still a toxic chemical for yeast. While fermenting, ethanol is excreted through the cell membrane by diffusion. In the beginning of the fermentation when the fermentation rate is at its highest, there can be a higher concentration inside the cell than on the outside due to faster production than diffusion as reported by D'Amore *et al*. [147]. The tolerance of yeast strains varies greatly and is closely related to the final amount they can produce by fermentation [12]. Average beer produced from a 12 °P wort has an ethanol concentration of about 5 % v/v. *Saccharomyces* yeast used to produce wine can be tolerant up to an ethanol concentration of 10-15 % v/v. It has been reported that *Saccharomyces* saké yeasts can ferment up to a total ethanol concentration of 20 % v/v [12]. Non-*Saccharomyces* genera such as *Brettanomyces* and *Zygosaccharomyces* have been reported to be as tolerant as *Saccharomyces* yeasts [148]. However the tolerance of yeast to ethanol is closely related to the total nutrition concentration, carbohydrate level, temperature and osmotic pressure. Many authors have reported various inhibitory effects of ethanol. Ethanol stress was described to be related to osmotic stress, leakage of amino acids and inhibition of transport systems. Ethanol is also a mutagen for the mitochondrial genome. As the ability to produce ethanol and to survive certain concentrations is closely related, an investigation of the tolerance of a potential brewing strains must be taken into account [12].

1.5 *Torulaspora delbrueckii* potential novel brewing yeast

The yeast species *T. delbrueckii* was first mentioned in relation to brewing by King and Richard Dickinson in 2000 [149]. They compared the biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. Monoterpene alcohols are flavor compounds of plant origin, which are also present in hops. In fact, linalool,

a monoterpene alcohol, has been reported to be a key contributing flavor compound to the hop aroma in beer [150]. King and Richard Dickinson reported a potential use of *T. delbrueckii* as brewing yeast since it showed the ability to transform monoterpene alcohols (e.g. nerol (fresh green aroma) into linalool (fresh green coriander aroma)), offering the potential to noticeably change hop flavor during fermentation [149]. *T. delbrueckii* was formerly investigated and described as a potential wine starter yeast as it showed good flavor forming and no off-flavors when added to wine fermentations [34, 151, 152]. Researchers reported evidence that some strains might have been domesticated in wine production, like *S. cerevisiae* has been for beer, over the past 4000 years [39]. As a result of some strains' good ability to produce desired flavors and to ferment well, it became the first commercially sold non-*Saccharomyces* starter culture for wine [34]. It was further reported that *T. delbrueckii* showed high tolerance towards ethanol and high sugar concentrations [153]. Utilization of different sugars was highly strain dependent as researchers found out when applying *T. delbrueckii* strains to bread dough [154] and performing different sugar utilization tests [126]. A strain-dependent high-maltose affinity was described by Alves-Araújo *et al*. in 2004 [140]. *T. delbrueckii* can be described as having been associated with human activities for many years [39]. The aspects of potential maltose and maltotriose fermentation, tolerance towards ethanol and high sugar concentrations as well as the potential ability to change hop flavor suggested *T. delbrueckii* had high potential as a model for the first characterization.

2 Results (Thesis publications)

2.1 Summary of results

The thesis publications are each summed up in the following paragraphs 2.2 to 2.5 with a description of authorship contribution followed by full copies of the publications. Table 1 gives an overall overview of the publications. Permission of publishers for the imprint of publications can be found in paragraph 5.4.

Table 1 Short overview of the four publications with title of the publication, major objective, applied method and main findings

Publication Title			
Publication 1 Review: Pure non - *Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical application	**Publication 2** Screening for the brewing ability of non-*Saccharomyces* yeast with *Torulaspora delbrueckii* as a model	**Publication 3** Optimization of beer fermentation with a novel brewing strain *Torulaspora delbrueckii* using response surface methodology	**Publication 4** A new approach for detecting spoilage yeast in pure bottom-fermenting and pure *Torulaspora delbrueckii* pitching yeast, propagation yeast, and finished beer
Major objective			
To summarize literature, conference papers and research on fermentations with pure cultures of non-*Saccharomyces* yeast in brewing.	To set up a screening system which will identify potential brewing yeast strains from genera besides *Saccharomyces*.	To optimize the fermentation parameters, temperature and pitching rate for one strain found with high fermentation potential, investigate the optimal propagation technique.	To implement a novel method for the detection of spoilage yeast in pitching yeast of *T. delbrueckii* or beer produced with *T. delbrueckii*
Applied methods			
Combining literature of the past decades, critical comparison of outcomes of differing studies.	Sugar utilization, ethanol and hop resistance tests, phenolic-off-flavor tests, Real-time polymerase Chain reaction, amino acid metabolism, secondary metabolite detection, trial fermentation	Design Expert Response Surface Methodology, trial fermentations, propagation system setup, secondary metabolite detection with HPLC, GC and trained panelists	Speedy Breedy pressure detection device, vitality measurement by acidification power test, Real-time polymerase chain reaction
Main findings/ conclusion			
Few *Brettanomyces, Saccharomycodes, Candida, Zygosaccharomyces* and *Torulaspora* species have been investigated. Different trial setups with highly varying parameters were conducted	The potential of fermenting beer wort and secondary metabolite production differs highly among strains. One strain of the species *T. delbrueckii* was found to offer great potential for the fermentation of wort into a beer of average alcohol content.	Optimal fermentation parameters for the *T. delbrueckii* T9 strain are 60 x 10^6 cells/mL pitching rate, 20 °C. 10 mg/L dissolved oxygen wort aeration is sufficient. High flavors of honey-, blackcurrant- and wine-like at differing fermentation temperatures.	Low concentration of spoilage yeast can be reliably detected in *T. delbrueckii* and bottom-fermenting pitching- and propagation yeast. Method also applicable to 37 °C positive wild yeast detection in lager beer and yeast.

Part 1	2.2 Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications

In recent years there has been increasing interest in the fermentation of brewer's wort by non-*Saccharomyces* yeast. Many groups of scientists have started to identify strains of non-*Saccharomyces* species that might contribute positively to beer flavor. Here a review of literature was compiled to summarize their work for alcohol free-, low alcohol- and average alcohol content beer. Before summarizing the different trials conducted with varying non-*Saccharomyces* yeast, the pathways of secondary metabolites relevant to beer flavor are explained. The authors added relevant thresholds to facilitate the amounts that the different trials showed. The large group of relevant secondary metabolites was split up into sulfuric compounds, undesirable carbonyl compounds, phenols, organic acids, higher alcohols, esters and monoterpene alcohols.

Almost all of the trials were conducted with varying parameters, giving very low comparability. However, the outcome of a beer fermentation can be strongly shaped by temperature, pH-value of the applied wort, pitching rate, original gravity, batch size and fermentation time. Each of these characteristics was first summed up for each yeast species in the review, giving the reader an overview before going into detail. Eight species were found to be used in different trials in literature: *Brettanomyces anomalus,* and *Brettanomyces bruxellensis, Candida tropicalis, Candida shehatae, Saccharomycodes ludwigii, Torulaspora delbrueckii, Pichia kluyveri, Zygosaccharomyces rouxii*. This publication sums up all the published trials performed with these eight species, showing how the particular investigations were performed. It discusses the results in between the different publications and the potential of the applied yeast strains for beer fermentation. Almost all species were found to be useful except for *Candida tropicalis* as this yeast has pathogenic properties. Both the *Brettanomyces* and *Torulaspora delbrueckii* species were suggested for beers with average alcohol content (approx. 5 % v/v). *Candida shehatae, Saccharomycodes ludwigii, Pichia kluyveri and Zygosaccharomyces rouxii* were suggested for low-alcohol beer production (approx. 0.5 % v/v alcohol).

Authors/Authorship contribution:

Michel M.: Literature search, writing, review conception and design; **Meier Dörnberg T.**: critical review of draft, discussion of data; **Jacob F.**: Supervised the project; **Methner F.**: Discussion of data; **Wagner R.**: Drafted article for English language and content; **Hutzler M.**: Critical revision, conception of review

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Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications

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Recently there has been increased interest in using non-*Saccharomyces* yeasts to ferment beer. The worldwide growth of craft beer and microbreweries has revitalised the use of different yeast strains with a pronounced impact on aroma and flavour. Using non-conventional yeast gives brewers a unique selling point to differentiate themselves. Belgian brewers have been very successful in using wild yeasts and mixed fermentations that often contain non-*Saccharomyces* yeasts. Historically, ancient beers and beers produced before the domestication of commonly used *Saccharomyces* strains most likely included non-*Saccharomyces* species. Given the renewed interest in using non-*Saccharomyces* yeasts to brew traditional beers and their potential application to produce low-alcohol or alcohol-free beer, the fermentation and flavour characteristics of different species of non-*Saccharomyces* pure culture yeast were screened for brewing potential (*Brettanomyces anomalus* and *bruxellensis*, *Candida tropicalis* and *shehatae*, *Saccharomycodes ludwigii*, *Torulaspora delbrueckii*, *Pichia kluyveri*, *Zygosaccharomyces rouxii*). Alcohol-free beer is already industrially produced using *S. ludwigii*, a maltose-negative species, which is a good example of the introduction of non-*Saccharomyces* yeast to breweries. Overall, non-*Saccharomyces* yeasts represent a large resource of biodiversity for the production of new beers and have the potential for wider application to other beverage and industrial applications. Almost all of the trials reviewed were conducted with varying fermentation parameters, which plays an important role in the outcome of the studies. To understand these impacts all trials were described with their major fermentation parameters. Copyright © 2016 The Institute of Brewing & Distilling

Keywords: non-*Saccharomyces*; alternative fermentation; beer; *Torulaspora delbrueckii*; *Saccharomycodes ludwigii*

Introduction

The use of non-*Saccharomyces* yeast as a pure starter culture for brewing has been growing rapidly in the past few years *(1–7)*. This is a result of an increased global interest in craft beer and larger brewers seeking different yeasts, often non-traditional yeasts, to innovate new beers. Wild and undomesticated non-*Saccharomyces* yeast strains can provide different aroma and flavour characteristics that result in alternative and new beer variations and styles *(6)*. However, since non-*Saccharomyces* yeasts represent undomesticated strains, their fermentation characteristics can be variable and they can affect the consistency and quality of the produced beers *(5,8)*. In contrast to non-*Saccharomyces* strains, the domestication of *Saccharomyces* yeasts for brewing has taken several millennia for brewers to produce consistent beers and control more desirable aromas, flavours and fermentation conditions *(1,5,9)*. Currently, brewers can choose from a wide range of different brewing yeasts and there is a good understanding about the brewing conditions (e.g. wort pH, malt interactions, temperature etc.) *(10)*. In terms of non-*Saccharomyces* there is very little knowledge of the fermentation parameters and produced aromas and flavours *(2)*. Most of the steps taken towards the domestication of the main brewing yeast *Saccharomyces* can now be performed much faster and with greater ease *(4,11)*, mostly because there is a greater understanding of the pathways of sugar and amino acid metabolism, as well as the formation of aroma-active substances such higher alcohols, esters, phenols, acids and monoterpenes *(4,12–16)*. However, despite our poor understanding of these characteristics for non-*Saccharomyces* yeasts, which can have a greater variation than *Saccharomyces* yeasts, there are many opportunities for brewers. For example, the non-*Saccharomyces* species can be used in the production of low-alcohol beer (0.5–1.2% v/v) and alcohol-free beer (<0.5% v/v) *(2)*. The strain *Saccharomycodes ludwigii* can produce alcohol-free beers with rich flavour profiles owing to its aroma production and low performance in fermenting maltose and maltotriose from wort *(17)*. Because of their potential for commercial viability, brewers and researchers have started to search for new yeasts in different environments using different techniques

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(18). Besides the opportunity of screening for pre-existing wild *Saccharomyces* and non-*Saccharomyces* species and strains, there is also the possibility of genetic modification either by selective breeding or adaptive evolution *(4,19)*. A review on the potential of *some* non-*Saccharomyces* yeast species for brewing has recently been published. It discusses results from wine fermentation and the potential use in brewing by secondary metabolites produced in mostly wine or media that contain sugar *(6)*. Our review focuses on reported brewing trials with the use of pure non-*Saccharomyces* starter cultures to produce normal gravity beers, low-alcohol and alcohol-free beers, with a major focus on the fermentation of brewers' wort, secondary metabolites and potential use of these species in breweries.

New and novel brewing strains can be discovered by judging the aroma and flavour characteristics of beer by experienced panellists but also by using physical and chemical analytical techniques to examine the flavour compounds that strains produce. Many significant flavour compounds that come from brewing yeast are mostly secondary metabolites. They are produced in relatively low quantities compared with the main fermentation products, which are ethanol and carbon dioxide *(14,20)*. Secondary metabolites can be divided into different categories and include sulphur-containing flavour compounds, undesirable carbonyl compounds, volatile phenols, organic acids, fusel alcohols, esters and monoterpene alcohols *(14,20–22)*. There are hundreds of different flavour-active substances that are yet to be described but in this paper some of the main categories are summarised before discussing the role of specific non-*Saccharomyces* yeast in production *(14)*. The categories play diverse roles in the impact and desirability of beer flavour. Some have a synergistic influence on the aroma although they are noticeably below flavour thresholds *(16,20,22,23)*. Since most of the trials summarised in this review analysed many different aroma active substances, a brief explanation as well as corresponding thresholds are provided.

Secondary metabolites relevant for beer flavour

Sulphuric compounds

Major sulphuric compounds produced by yeast during beer fermentation include sulphur dioxide and hydrogen sulphide *(24,25)*. Brewers are very familiar with sulphur dioxide as it plays a large role in flavour stability, mainly by acting as an antioxidant in the finished beer to considerably increase shelf life. Sulphur dioxide is usually produced in quantities of $<$10 g/L as a side or feedback inhibition product of amino acid anabolism *(24,26)*. Also, sulphur dioxide has a sulphurous odour threshold of 2.5 mg/L, which is a desired positive characteristic in some bottom-fermented beers *(26,27)*. In comparison, hydrogen sulphide is an undesirable compound because it has a high potential to mask other positive beer flavours and has a very low threshold of 0.005 mg/L, where it is perceived as a rotten-egg smell *(28,29)*. Hydrogen sulphide is mostly produced during the cell maturation cycle but is later assimilated in the budding cycle *(25)*.

Undesirable carbonyl compounds

The key undesirable carbonyl compounds in beer are acetaldehyde and vicinal diketones. Of these, diacetyl plays a major role in beer flavour owing to its mostly undesirable flavour and very low flavour threshold and is similar to 2,3-pentanedione, another unwanted compound *(22,30)*. Acetaldehyde is predominantly produced during the growth phase of yeast, as a result of sugar metabolism, and in the subsequent fermentation most of it is converted to ethanol *(31)*. It has a mostly undesirable green apple-like or grassy flavour with a threshold of 10 mg/L *(22)*. Diacetyl adds a buttery flavour to beer if found above its threshold of 0.1–0.15 mg/L *(22,32)*. Diacetyl is a by-product of the amino acid anabolism of valine, formed during a four-step reaction from glucose. Diacetyl is then taken up by the yeast again later during maturation and reduced to 2,3-butanediol, which does not have an undesirable flavour *(30,33)*. The compound 2,3-pentanedione is known for its toffee-like flavour similar to diacetyl with a threshold of ~0.9 mg/L *(34)*. It is produced as a by-product in the synthesis of the amino acid isoleucine in the mitochondria of yeast cells *(35)*. For more detailed information, an extensive review on diacetyl and 2,3-pentanedione was published in 2013 by Krogerus and Gibson *(30)*.

Phenols

Most flavours that come from yeast-produced phenols are referred to as 'phenolic off-flavours'. However, in some beer types such as Belgian lambic, Belgian Abbey-style ales and German wheat beer these flavours are desired. The most common odours coming from these substances include clove-like, smoky, spicy, medicinal and burnt aromas *(36,37)*. The most common of these are 4-vinylguaiacol, 4-vinylphenol, 4-ethylguaiacol, 4-ethylphenol, 4-vinylsyringol, styrene, eugenol and vanillin *(38)*. The synthesis of these compounds depends on the yeast species as well as on the presence of precursors in the wort. The precursors include phenolic acids with a high flavour threshold such as ferulic, coumaric and cinnamic acid, which come from malt *(38,39)*. Beers fermented with *S. cerevisiae* will mostly contain 4-vinylguaiacol and 4-vinylphenol, since they can only perform decarboxylation of phenolic acids. However, species such as *Brettanomyces* sp. are able to reduce some of the compounds to 4-ethylguaiacol and 4-ethylphenol *(38)*. The thresholds for these volatile phenols are low: 4-ethylphenol, 0.9 mg/L (phenolic aroma, astringent); 4-ethylguaiacol, 0.13 mg/L (phenolic aroma, sweet); 4-vinylguaiacol, 0.3 mg/L (phenolic aroma, bitter, clove); and 4-vinylphenol 0.2 mg/L (phenolic aroma, smoky) *(34,40)*.

Organic acids

The large group of organic acids contributes to the flavour of beer and to the final total acidity (i.e. pH) together with inorganic acids. Organic acids can be divided into two major classes, which include volatile and non-volatile acids *(41)*. The main volatile organic acids that occur in beer are acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, caprylic, capric and lauric acid. If they are present in high concentrations they contribute a sour and salty flavour to beer and can also contribute to off-flavours such as cheesy and sweaty *(22,42–44)*. Quantitatively, the volatile acids that impact flavour the most are acetic, caprylic, capric and lauric *(43,45)*. Acetic acid, the predominant molecule in vinegar, has a threshold of 175 mg/L while caprylic acid has a much lower threshold of 15 mg/L and is described as goaty. Capric acid is described as waxy with a threshold of 10 mg/L. Lauric acid is described as soapy when reaching a threshold of 6.1 mg/L *(22)*. The main non-volatile acids in beer produced by yeasts that impact the flavour and aroma include (threshold indicated): oxalic acid

(500 mg/L, salty, oxidised), citric acid (400 mg/L, sour), malic acid (700 mg/L, apple) , fumaric acid (400 mg/L, sour, acidic), succinic acid (220 mg/L, acidic), lactic acid (400 mg/L, sour, acidic) and pyruvic acid (300 mg/L, salty, forage) *(22,43–47)*. The production of these compounds is dependent on the yeast strain with most of these acids being by-products of glycolysis, the citric acid cycle, amino acids and fatty acid metabolism *(47)*.

Higher alcohols

Besides the main alcohol component ethanol there are many higher or so-called fusel alcohols that contribute significantly to the flavour of beer. Higher alcohols can contribute floral, fruity or herbal aromas depending on their synergistic effects with other flavour-active compounds. The most important higher alcohols in beer include *n*-propanol, isobutanol, isoamyl alcohol and 2-phenylethanol *(14,22,48)*. The threshold of *n*-propanol has the highest value of 600 mg/L, which contributes to a sweet alcoholic flavour *(48)*. Iso-butanol and amyl alcohol have solvent-like aromas but differ in their thresholds of 100 and 50–70 mg/L, respectively. Isoamyl alcohol and 2-phenylethanol have more fruity aromas, whereas isoamyl alcohol displays more banana, alcoholic flavours at a threshold of 50–65 mg/L. The compound 2-phenylethanol has a gummy bear and rose-like flavour at a threshold of 40 mg/L *(22,48)*. Yeast produces higher alcohols as a by-product of amino acid metabolism and catabolism. During catabolism amino acids are taken up by yeast cells and transaminated by four transaminase enzymes *(49)*. The resulting product is an α-keto acid, which then undergoes an irreversible reaction to form a higher alcohol. This reaction was first described as the Ehrlich pathway *(50,51)*. Metabolism of amino acids starts with a carbohydrate, which is modified to an α-keto acid. This acid is then transaminated into the corresponding amino acid and higher alcohols are formed by the process of decarboxylation and reduction of these α-keto acids *(51)*. For more detailed information, a full review of higher alcohols in beer has been published in 2014 by Pires et al. *(14)*.

Esters

The most important flavour-active substances within the group of higher alcohols are esters despite their low concentrations relative to other flavour-active compounds *(14,20)*. They contribute a wide range of fruity flavours to the composition of fermented beverages. These aroma-active esters can be divided into acetate esters and medium-chain fatty acid ethyl esters *(14,20)*. Acetate esters are synthesised from a higher alcohol or ethanol with acetic acid and have the highest concentration of flavour-active esters in beer. The most important of these esters in beer are ethyl acetate (solvent aroma with a threshold of 33 mg/L), isoamyl acetate (banana aroma with a threshold of 1.6 mg/L), isobutyl acetate (fruity and sweet aroma with a threshold of 1.6 mg/L) and phenyl ethyl acetate (rose, apple and honey aroma with a threshold of 3.8 mg/L) *(22,48,52)*. Medium-chain fatty acid ethyl esters are formed, as the name implies, from a medium-chain fatty acid and an ethanol radical. Two of these esters are key to beer flavour, for example ethyl hexanoate (ethyl caproate) with a threshold of 0.23 mg/L produces apple and aniseed aromas, and ethyl octanoate (ethyl caprylate) with a threshold of 0.9 mg/L produces a sour apple aroma *(14,20,22)*. There are some extensively written publications that can be reviewed for further details on esters and their pathways *(14,16,20)*.

Monoterpene alcohols

These substances, which are derived from plants, contribute highly floral aromas to the flavour of beer, juice and wine *(53–55)*. In the case of beer they are derived from hops and five of these substances are present at noticeable concentrations *(53,54)*. These five monoterpene alcohols that are critical to flavour are (threshold from literature and aroma indicated): linalool (5 μg/L, lavender), α-terpineol (2 mg/L, lilac), β-citronellol (8 μg/L, lemon, lime), geraniol (6 μg/L, rose) and nerol (0.5 mg/L, rose, citrus) *(34,53,54)*. Yeast is able to transform these compounds and shift the ratios in between the five main occurring monoterpene alcohols, which can lead to a change in the beer's hop flavour after fermentation *(55)*. Furthermore, these compounds can also be present in wort in glycosidically bound forms resulting in an aroma inactive stage *(21,56)*. Some yeast species have the enzyme glucoside hydrolase, which allows them to release the monoterpene, which changes or enhances the hop flavour *(21,57)*.

Non-*Saccharomyces* yeast fermentation

The following section discusses non-*Saccharomyces* yeast characteristics derived from impure wort fermentations as well as a summary of fermentation information from literature.

Non-*Saccharomyces* yeasts used as pure starter cultures for the fermentation of beer wort

1. *Torulaspora delbrueckii* is known from the wine industry for adding more fruitiness to wine flavour *(58)*.
2. *Brettanomyces anomalus* is well known to the beverage industry as a spoilage yeast but it also partially participates in mixed fermentations of lambic and gueuze beers *(59)*.
3. *Brettanomyces bruxellensis* is known for the horsey flavour and acidity it adds to mixed fermentations of lambic and gueuze beers *(59)*. It is further known as the main spoilage yeast for wine *(60)*.
4. *Candida shehatae* is known to ferment xylose for ethanol production *(61)*.
5. *Candida tropicalis* is known from mixed fermentations of traditionally fermented beverages from African countries *(62)*.
6. *Saccharomycodes ludwigii* does not ferment the main wort sugars maltose or maltotriose and therefore produces low alcohol levels but highly desirable flavours in beer *(17)*.
7. *Zygosaccharomyces rouxii* is capable of consuming ethanol under aerobic conditions and produces actively desired flavour compounds for producing low-alcohol beers *(63)*.
8. *Pichia kluyveri* is capable of fermenting only glucose in brewer's wort but produces high concentrations of desirable flavour compounds and very low ethanol quantities *(64)*.

One of the most important characteristics for brewers is the sugar utilisation of the main wort sugars by yeast (glucose, fructose, sucrose, maltose, maltotriose) *(65,66)*. It determines the ability to ferment wort, produce beer economically, and establishes the final beer type (low-alcohol beer, average original gravity beer, etc.). For each species described in this review a type strain (according to CBS database http://www.cbs.knaw.nl/Collections/, 28 June 2016) and general species data (according to Kurtzman *(67)*) are given with their ability to ferment different sugars (Table 1). It should be noted that maltotriose is not shown in the table because the data could not be found for this particular sugar.

Table 1. Literature and type strain sugar fermentation of the different yeast strains used in the following studies

Taxonomy	Literature/ type strain	Fermentable sugar						
		Glucose	Lactose	Galactose	Raffinose	Sucrose	Trehalose	Maltose
Torulaspora delbrueckii	*(85)*	+	–	v	v	v	v	v
	CBS[a] 1146	+	w	+	w	+	w	+
Brettanomyces anomalus	*(85)*	+	v	+	v	+	v	v
	CBS 8139	+	w	+	–	+	w	w
Brettanomyces bruxellensis	*(85)*	+	v	+	v	+	v	v
	CBS 74	w	–	–	–	+	+	+
Candida shehatae	*(85)*	+	v	+	v	+	v	v
	CBS 5813	+	–	+	–	–	+	+
Candida tropicalis	*(85)*	+	–	+	–	v	+	+
	CBS 94	+	–	+	–	+	w	+
Saccharomycode ludwigii	*(85)*	+	–	–	+	+	–	–
	CBS 821	+	–	–	+	+	n.a.	–
Zygosaccharomyces rouxii	*(85)*	+	–	–	–	v	w	w
	CBS 732	+	n.a.	n.a.	n.a.	n.a.	n.a.	+
Pichia kluyveri	*(85)*	+	–	–	–	–	–	–
	CBS 188	+	–	–	–	–	–	–

w, Weak; v, variable; +, positive; –, negative; n.a., not analysed.
[a]CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

The following is a description of yeast strain origin, summary of brewing trials, and known fermentation parameters.

Torulaspora delbrueckii. *Torulaspora delbrueckii* was first suggested as a potential brewing yeast by King and Dickinson *(55)*. They discovered that, although this particular *T. delbrueckii* strain came from the wine industry, it was able to transform the aroma of hops by noticeably changing the monoterpene alcohol composition. It was formerly described under the *Saccharomyces* genus as *Saccharomyces rosei* and *S. roseus (67)*. In the wine industry it was used in mixed fermentations for producing wine and sparkling wine *(68)*. For wine it increased the sensorial complexity and showed very low production of so-called off-flavours such as phenols or sulphuric compounds *(69–72)*. It produced strong fruity notes and was able to survive at high ethanol concentrations, which was surprising for a non-*Saccharomyces* genus *(67,68)*. It has also been studied in the context of bread making; high concentrations of salt and sugar are lethal for some yeasts that occur in most bread doughs, and *T. delbrueckii* showed a high osmotic tolerance towards the sugar and salt in baking doughs. It was therefore considered a novel baking yeast. Since high osmotic tolerance is a challenge for high-gravity brewing, this might be a good yeast option *(73)*. Furthermore, it showed high vitality after being deep frozen in dough *(74–76)*. Table 2 is a summary of all the trials performed with pure *T. delbrueckii*, followed by an explanation of the procedures and outcomes of the different studies.

Table 2. Summary of all trial fermentations performed with different strains of *T. delbrueckii*, wort attributes, fermentation conditions and source of publication

Strain code	Wort attributes			Fermentation parameters					Source
	pH	OG (°P)	IBU	Temperature (°C)	Pitching rate (cells/mL) × 10^6	Batch size (L)	Time	Storage	
Td 28	NM	11	NM	20	NM	3.5	204 h	7 days 25 °C, 14 days 10 °C	*(77)*
LTQB7	4.95	16	NM	28	NM	1.2	240 h	None	*(78)*
C-05716	u	15	NM	20	NM	0.065	90 h	None	*(79)*
DiSVA 254	5.47	12.7	NM	20	NM	1.5	456 h	7–10 days at 18–20 °C in 500 mL bottles plus 5 g sucrose	*(80)*
T6, T9, T10, T11, T13, T15, T17, T18, T19, T20	5.2	12	0	27	15	2	168 h	None	*(81)*
TD-A01, TD-B03	5.3	12,2	0	20	NM	100	250–300 h	Bottle conditioned	*(84)*

NM, Not mentioned; OG, original gravity; IBU, international bitterness units.

Tataridis et al. *(77)* used several webpages (without scientific references) as their inspiration for using *T. delbrueckii* as a yeast to produce German wheat beer. They pitched 25 g of dried *T. delbrueckii* into 3.5 L of beer wort at an original gravity of 11°P, and fermented it at 20 °C for 204 h. It was compared with a top-fermenting strain of *S. cerevisiae* under the same conditions. After fermentation the beer was stored at 25 °C for 7 days followed by 14 days of maturation at 10 °C. The beer produced was analysed for final gravity and turbidity, and evaluated for flavour and aroma by a panel of expert brewers. It was found to have a final gravity of 3.0°P, a high ester content, with aromas of rose, bubblegum and banana, and was preferred by most panellists over the control top-fermented beer.

Pozippe et al. *(78)* examined a *T. delbrueckii* strain for its growth under different stress conditions as well as the volatile profile of a 16°P beer wort fermented at 28 °C. In contrast to the report of Tataridis et al. *(77)*, the fermentation was described as slow (about 10 days), which seems obvious when comparing the original gravity of the two different trials (11 to 16°P); however, no data of the pitching rate was published, which is a potential confounding factor. The beers that were produced had high levels of acetaldehyde, high concentrations of fusel alcohols and an ethanol concentration of 5% (v/v) (Table 3).

Gibson et al. *(79)* investigated a *T. delbrueckii* strain for the production of 4-vinylguaiacol, 3-methylbutanol, 3-methylbutylacetate, ethyl caprylate, 2-phenylethanol, 2-phenylethylacetate and ethyl decanoate. They fermented a 15°P wort pitched with 0.02 g/L of fresh yeast at 20 °C for 90 h. Concentrations of flavour compounds were compared with a control ale strain. The *T. delbrueckii* strain that was used did not show any high concentrations of the investigated flavour compounds except for 3-methylbutanol (solvent-like flavour) and was disregarded for further research.

Canonico et al. *(80)* screened 28 strains of *T. delbrueckii* for their possible use in beer wort fermentation. They first analysed sugar utilisation for main wort sugars and then selected one strain, isolated from papaya leaves in Cameroon, which they used for further fermentations. For these fermentations they used an American amber ale wort with an original gravity of 12.7°P and a pH value of 5.47. The trial fermentations were carried out with 1.5 L of wort, which was fermented at 20 °C for 19 days. After the first fermentation the beer was transferred to 500 mL bottles and 5 g of sucrose was added for the second fermentation, which was carried out at 18–20 °C for 7–10 days. They then analysed the volatile acidity, pH value, esters, higher alcohols and ethanol concentrations. Furthermore, they performed sensorial analyses using six trained panellists who judged the beers by aromatic notes (i.e. floral fruity, etc.) and main structural features (i.e. flavour, bitterness, etc.). The beers produced showed a low real attenuation of 37% with high amounts of acetaldehyde (7.5 mg/L). Owing to the low attenuation the ethanol concentration was only 2.66% (v/v) and had a low amyl-alcohol content of 7.98 mg/L (see Table 4).

The panellists described the beers as fruity and citric with a full body. The author therefore concluded that *T. delbrueckii* could be used for producing beers with a pleasant and aromatic taste. Owing to the low attenuation of the finished beers, the author suggested using *T. delbrueckii* to produce low-alcohol beers *(80)*.

Michel et al. *(81)* studied 10 *T. delbrueckii* strains from different habitats. They investigated sugar utilisation, hops and ethanol resistance, their propagation ability, amino acid metabolism, anabolism and phenolic off-flavour-forming capability. Furthermore, they conducted trial fermentations with beer wort (12°P) at 27 °C, which were analysed for extract reduction, pH drop, yeast concentration in suspension of supernatant and fermentation by-products. One strain was identified as being able to ferment the beer wort to an ethanol concentration of 4% v/v with a desirable fruity and floral aroma. It produced high amounts of 2-phenylethanol (23.7 mg/L) as well as high amounts of amyl alcohols 64.83 mg/L. Furthermore, two strains were found to be suitable for producing low-alcohol beer owing to their inability to ferment maltose and maltotriose but still produced good flavour (see Table 5) *(81–83)*.

Tatraidis et al. *(84)* fermented two different worts, one with 100% malt (12.2°P, pH 5.3) and one with 60% malt wort and 40% glucose added. They used two *T. delbruckii* strains and one reference ale strain (*S. cerevisiae*). The fermentation of the 100% malt wort was carried out at 20 °C and one strain TD-A01 showed slightly lower fermentation speed compared with the reference strain. The second strain, TD-B03, also showed very low fermentation speed but only half of the apparent attenuation (36%). TD-A01 produced an ethanol concentration of 4.2% v/v whereas TD-B03 produced 2.34% v/v. The authors analysed some volatile compounds, acidity and volatile acidity of the final beers. The beer

Table 3. Volatile compounds measured in finished beer after fermentation with *T. delbrueckii* strain LTQB7 *(78)*

Volatile compounds	Acetaldehyde	Ethyl acetate	1-Propanol	Isobutanol	Isoamyl alcohol	Acetic acid	Higher alcohols
LTQB7	47.9 mg/L	16.2 mg/L	22.4 mg/L	23.3 mg/L	69 mg/L	93.4 mg/L	114.8 mg/L

Table 4. Beer attributes, main secondary metabolites and main volatile compounds of the fermentation performed with pure *T. delbrueckii* strain DISVA 254 *(80)*

Beer attributes		Main fermentation by-products (mg/L)		Main volatile compounds (mg/L)	
pH value	4.56	Acetaldehyde	7.5	Ethyl butyrate	0.168
Final gravity (°P)	7.51	Ethyl acetate	3.46	Isoamyl acetate	0.134
Ethanol (% v/v)	2.66	*n*-Propanol	15.41	Ethyl hexanoate	0.031
Attenuation (%)	45.09	Isobutanol	7.98	Ethyl octanoate	0.006
Volatile acidity (g/L)	0.15	Amyl alcohol	3.82	Butyric acid	0.074
FAN (mg/L)	174.58	Isoamyl alcohol	32.79	2-Phenyl ethanol	6.52

Table 5. Fermented sugars, beer attributes and secondary metabolites of the 10 *T. delbrueckii* strains used by Michel et al. *(81)*

Yeast		T6	T9	T10	T11	T13	T15	T17	T18	T19	T20
Fermented sugars (%)	Glucose	96.6	96.2	97	96.6	97.3	95.5	94.5	95.4	94.7	89.6
	Fructose	93.2	92.3	91.54	88	91.6	90.2	84.5	96.4	93.6	88.1
	Sucrose	82.4	86.4	79	95	84.6	75.2	78.3	72	73.7	84.7
	Maltose	3.3	94.8	5.8	6	1.8	0.3	0.9	2.5	0.7	0.3
	Maltotriose	3	58.9	1.6	4.2	0.5	1.3	2.4	0.1	0.1	3.6
Beer attributes	pH value	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2
	Ethanol (% v/v)	0.87	4	0.83	0.87	0.89	0.94	0.89	0.87	0.9	0.91
Secondary	Ethyl acetate	3.83	23.4	2.23	4.56	5.33	2.76	5.96	3.76	3.9	4.36
metabolite (mg/L)	Amyl alcohols	28.03	64.83	25.26	25.93	29.1	24.1	28.36	20.73	15	18.6
	Diacetyl	0.31	0.43	0.13	0.14	0.1	0.15	0.12	0.15	0.2	0.14
	Decanoic acid	2	0.02	0.67	0.49	1.13	0.08	0.67	2.06	1.07	0.35
	2-Phenylethanol	12.83	23.7	15.83	8.13	13.9	11.8	8.93	5.4	5.03	5.26
	n-Propanol	4.83	17.06	3.03	4.9	4.13	5.7	3.66	2.93	2.73	2.33
	Isobutanol	3.46	19.96	2.36	4.46	3.56	2.1	3.3	1.36	2.8	4
	Hexanoic acid	0.2	0.07	0.16	0.18	0.2	0.13	0.13	0.23	0.13	0.13
	Octanoic acid	0.59	0.11	0.36	0.49	0.57	0.33	0.42	0.74	0.39	0.3

fermented with TD-A01 showed high amounts of isoamyl acetate (15.6 mg/L) and 2-phenylethyl acetate (29.1 mg/L) as well as the comparatively lowest volatile acidity (volatile organic acids) of 0.14 g/L compared with the other TD-B03 strain (0.17 g/L) and the brewing strain (0.21 g/L). TD-B03 produced far fewer esters as well as a high amount of isobutyric acid (17.9 mg/L). Twelve panellists judged the beers and described the *T. delbrueckii* fermented beers as highly estery and fruity as well as being full bodied *(84)*.

Overall it can be said that the fermentation performance of *T. delbrueckii* strongly depends on the strain. The overall speed of fermentation seems to be slower than that of usual *S. cerevisiae* brewing strains as shown by Tataridis, Canonico and Michel *(80–84)*. *T. delbrueckii*, however, ferments both, high and medium original gravity worts with a high production of higher alcohols and esters *(77,78,81)*. The formation of ethanol in the produced beers varied from 0.8 to 4%(v/v) which is due to the fact that some strains will not ferment all wort sugars *(80,81)*. Most of the above studies described an overall fruity flavour *(77,80)*. In general, some of the strains seemed to indicate a potential for use in brewing.

Brettanomyces anomalus. *Brettanomyces anomalus* can be found in mixed fermentations of gueuze and lambic beers and is well known to the beverage industry as a spoilage yeast in wine and soft drinks *(59,85)*. Most *B. anomalus* strains can ferment the main sugars present in beer wort as seen in Table 1. As a result of β-glucosidase activity, *B. anolamus* can hydrolyse glucoside-bound monoterpenes, which are present in many fruits and also in brewers' wort that comes from hops *(57)*. The splitting of these bonds releases monoterpenes, changing them into active flavour compounds. This could increase or modify the hop aroma because many of the released monoterpenes, such as linalool, are the key aroma substances from hops *(55)*. The positive influence of *B. anolamus* on wine aroma was investigated even though most winemakers still reject this yeast because it can produce a mousy off-flavour *(86,87)*. However, it was reported that it can also add positive sensorial complexity to the flavour of wine *(86)*. The importance for brewers is that it can metabolise some of the acids that are present in brewers wort such as *p*-coumaric and ferulic acid, and form phenolic aroma compounds such as 4-vinylguaiacol or 4-vinylphenol *(36,88)*. Table 6 shows a summary of all the trials conducted with pure *B. anomalus* followed by an explanation of the process and the outcome of the different studies.

Yakobson *(89)* used two *B. anomalus* strains (WY 5151 and WLP 645) to ferment wort with an original gravity of 12°P at 21–22 °C. He investigated the impact of pitching rate and acidification with varying amounts of lactate on attenuation, volatile flavour compounds and pH value of the final product. WY 5151 showed a rise in volatile flavour compounds with increasing pitching rates. Both strains showed almost no production of ethyl caproate and ethyl caprylate. The production of ethyl acetate was described as being very low but increased with increasing lactic acid addition. Further increased lactic acid correlated to a rise in higher alcohol production, a result also observed by Yakobson *(89)*. Furthermore, a significant change in attenuation could be observed by adding lactic acid and fermenting with WY 5151 resulting in attenuation ranging from 34.39% to 70.43% (Table 7).

Lentz et al. *(5)* used two different worts (12 and 14°P) to analyse the potential of three strains of *Brettanomyces anomalus* to ferment wort into beer. Fermentations were carried out at 20 °C for 4 weeks followed by 4 weeks of maturation. All strains fermented both worts to about 70% apparent attenuation. Lentz et al. *(5)* also reported that all strains showed robust growth up to an ethanol concentration of 12.0% (v/v), which they tested in MYPG medium. Finished beers were analysed by trained panellists and described with phenolic descriptors that included plastic, solvent and burnt or smoky. However, some were additionally described as fruity and desirably complex. All three strains were considered to have brewing potential.

Gamero et al. *(90)* used one strain of *B. anomalus* (CBS 77) to ferment commercial beer wort at 12 °C and conducted an ester analysis. The strain showed very low production of esters except for isoamyl acetate (about 2 mg/L). Total ester content did not exceed 5 mg/L.

Brettanomyces anomalus can produce fruity as well as phenolic flavours in beer fermentation *(5,89)*. The fermentation times in the above studies are longer than with a commercial *Saccharomyces* brewing strain. Temperatures for fermentation were mostly around 20 °C, which might be more optimal, and Gamero et al. *(90)* reported very low ester production at 12 °C *(5,89,90)*. Lower initial wort pH by adding lactate increased the production of flavour-

Table 6. Summary of all trial fermentations conducted with *B. anomalus* with strain codes, wort attributes, fermentation attributes, and source of publication

Strain codes	Wort attributes			Fermentation parameters					Source
	pH	OG (°P)	IBU	Temperature (°C)	Pitching rate (cells/mL) × 10^6	Batch size (L)	Time	Storage	
WY 5151	4.95	12	22	21–22	18	1.8	35 days	None	*(89)*
WLP 645, WY 5151	4.95	12	22	21–22	12	1.8	35 days	None	
WY 5151	4.95	12	22	21–22	6	1.8	35 days	None	
WLP 645, WY 5151	4.55	12	22	21–22	12	1.8	35 days	None	
WLP 645, WY 5151	4.05	12	22	21–22	12	1.8	35 days	None	
WLP 645, WY 5151	3.75	12	22	21–22	12	1.8	35 days	None	
WLP 645, WY 5151	3.08	12	22	21–22	12	1.8	35 days	None	
RS01, CS01, Ej02	NM	12	NM	20	NM	NM	28 days	None	*(5)*
RS01, CS01, Ej02	NM	14	NM	20	NM	NM	28 days	None	
CBS 77	NM	n.a.	NM	12	NM	0.2	NM	None	*(90)*

NM, Not Mentioned; OG, original gravity; IBU, international bitterness units.

Table 7. Summary of the beer attributes and secondary metabolites of fermentations performed with *B. anomalus* with varying pitching rates and lactic acid addition *(89)*

Yeast strain	WY 5151							WLP 645				
Cell count (cells/mL × 10^6)/ lactic acid (mg/L)	6/0	18/0	12/0	12/100	12/500	12/1000	12/3000	12/0	12/100	12/500	12/1000	12/3000
Apparent attenuation (%)	n.a.	n.a.	34.39	23.43	32.96	48.86	70.43	20.9	17.47	18.98	24.33	25.64
Final pH value	n.a.	n.a.	4.2	4.14	3.86	3.55	3.15	4.32	4.34	4.04	3.78	3.36
Acetaldehyde (mg/L)	1.44	1.35	1.16	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl acetate (mg/L)	1.67	3.86	2.68	6.78	12.05	17.95	20.99	1.26	2.03	2.92	1.6	1.56
Ethyl lactate (mg/L)	0.45	0.56	0.59	1.48	3.9	7.56	35.99	0.28	1.93	3.34	4.95	15.42
Ethyl butyrate (mg/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0	n.a.	n.a.	n.a.	n.a.
Ethyl caproate (mg/L)	0.03	0.05	0.04	0.15	0.23	0.17	0.13	n.a.	0.07	0.08	0.08	0
Ethyl caprylate (mg/L)	0.19	0.24	0.22	0.83	1.22	0.76	0.22	0.72	0.3	0.44	0.13	0
n-Propanol (mg/L)	1.04	1.51	1.12	n.a.	n.a.	n.a.	n.a.	0.59	n.a.	n.a.	n.a.	n.a.
Isobutanol (mg/L)	1.44	2.25	1.63	n.a.	n.a.	n.a.	n.a.	0.7	n.a.	n.a.	n.a.	n.a.
2-Methylbutanol (mg/L)	0.49	0.81	0.6	n.a.	n.a.	n.a.	n.a.	0.27	n.a.	n.a.	n.a.	n.a.
3-Methylbutanol (mg/L)	1.72	2.79	2.14	n.a.	n.a.	n.a.	n.a.	0.91	n.a.	n.a.	n.a.	n.a.
4-Vinylguaiacol (mg/L)	0.098	0.074	0.085	0.07	0.01	0	0	0.04	0.06	0.05	0.04	0.03
4-Vinylphenol (mg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diacetyl (mg/L)	0.019	0.048	0.038	n.a.	n.a.	n.a.	n.a.	0.012	n.a.	n.a.	n.a.	n.a.
2.3-Pentanedione (mg/L)	0.004	0.007	0.007	n.a.	n.a.	n.a.	n.a.	0.003	n.a.	n.a.	n.a.	n.a.

n.d., Not detected; n.a., Not analyzed.

active compounds *(89)*. However, the fermentation performance with respect to extract reduction and ethanol production seems rather low considering the fermentation period and high temperatures. However, it might be interesting for the production of some special beers.

Brettanomyces bruxellensis. *Brettanomyces bruxellensis* has a long tradition of being used in the mixed fermentation of lambic and gueuze beers in Belgium *(59,91)*. It is also very well known from being used in Berliner weißbier and some sour English ales *(59,92)*. The first *Brettanomyces* species was described by Claussen *(59,93)*. *B. bruxellensis* produces flavours described as mousy and horsey but also fruity and phenolic *(59)*. Sometimes the flavour is also described as metallic or bitter *(94)*. It was reported that *B. bruxellensis* is able to produce many ethyl esters such as ethyl acetate *(95)*. In addition, *B. bruxellensis* is a positive phenolic off-flavour, which is described in the above section 'Secondary metabolites relevant for beer flavour, phenols'. It has a high ethanol tolerance up to concentrations of 15% (v/v) *(96)* and can ferment some of the main wort sugars (see Table 1). Furthermore, it can utilise complex carbohydrates such as maltotetraose and maltopentaose, which leads to super attenuation but could also contribute to a dry mouthfeel *(97)*. A detailed review on *B. bruxellensis* has recently been published by Crauwels et al. *(98)*. Table 8 shows a summary of all the trials with pure *B. bruxellensis* followed by an explanation of the process and the outcome of the different studies.

Methner *(92)* investigated the pure culture use of *B. bruxellensis* by fermenting a 7°P beer wort (0.65 IBU) at a pitching rate of 13.3 × 10^6 cells/mL at 25 °C for 4 weeks. The wort was produced for Berliner weißbier, which is very low in IBU and low in OG. The beer was then stored for 4 weeks at 15 °C. It was analysed for esters, volatile and non-volatile fatty acids. In addition, he pitched

Table 8. Summary of all trial fermentations performed with *B. bruxellensis* with strain codes, wort attributes, fermentation attributes and source of publication

Strain codes	Wort attributes			Fermentation parameters					Source
	pH	OG (°P)	IBU	Temp. (°C)	Pitching rate (cells/mL) × 10^6	Batch size (L)	Time	Storage	
B. bruxellensis (no strain code indicated)	NM	7	0.65	25, 27	13.3, 1	NM	28 days	4 weeks at 15 °C	*(92)*
WY 5526, CMY 001, BSI-Drie	4.95	12	22	21–22	18	1.8	35 days	None	*(89)*
WY 5112, WY 5526, CMY 001, BSI-Drie, WLP 650, WLP 653	4.95	12	22	21–22	12	1.8	35 days	None	
WY 5526, CMY 001, BSI-Drie	4.95	12	22	21–22	6	1.8	35 days	None	
WY 5112, WY 5526, CMY 001, BSI-Drie, WLP 650, WLP 654	4.55	12	22	21–22	12	1.8	35 days	None	
WY 5112, WY 5526, CMY 001, BSI-Drie, WLP 650, WLP 655	4.05	12	22	21–22	12	1.8	35 days	None	
WY 5112, WY 5526, CMY 001, BSI-Drie, WLP 650, WLP 656	3.75	12	22	21–22	12	1.8	35 days	None	
WY 5112, WY 5526, CMY 001, BSI-Drie, WLP 650, WLP 657	3.08	12	22	21–22	12	1.8	35 days	None	
Bc02, Bc07	NM	10	NM	20	NM	NM	28 days	None	*(5)*
Bc02, Bc07	NM	12	NM	20	NM	NM	28 days	None	
Bc02, Bc07	NM	14	NM	20	NM	NM	28 days	None	
LTQB6	NM	16	NM	28	NM	1.2	10 days	None	*(78)*
CBS 2796	NM	NM	NM	12	NM	0.2	NM	None	*(90)*
BSI-Drie, BBY011, BBY017, BBY024, BBY026, BBY028, EBY010, PEST III, PEST IV	NM	12.5	NM	15, 22.5	12	0.07	28 days	NM	*(99)*

NM, Not Mentioned; OG, original gravity; IBU, international bitterness units.

1 × 10^6 cells/mL into the same wort and fermented it semi-aerobically for 2 weeks at 27 °C. The second batch was analysed for higher alcohols. For the first trial he reported a slightly higher quantity of ethyl acetate (25 mg/L) and a very low quantity of isoamyl acetate. The total esters was 37.6 mg/L. Furthermore, he reported high quantities of acetic acid (198 mg/L) and isovaleric acid (3.9 mg/L). In the second trial, Methner found high amounts of isovaleric acid (14 mg/L) as well as comparable amounts of higher alcohols similarly to a control beer fermented with *S. cerevisiae*. Almost all of the volatile acids increased as the temperature was increased to 27 °C (Table 9).

Yakobson *(89)* investigated six different strains of *B. bruxellensis* for their use in pure culture fermentation of beer wort. He first investigated the influence of three different pitching rates (6 × 10^6, 12 × 10^6 and 18 × 10^6 cells/mL) to ferment a beer wort with three different strains at a pH value of 4.95 and an original gravity of 12°P (Table 11). Fermentations were carried out in 1.8 L batches for 35 days at 21–22 °C. Furthermore, different pH values of the wort were investigated by adding 100, 500, 1000 and 3000 mg/L of lactic acid, pitching all six strains into individual batches of 1.8 L at 12 × 10^6 cell/mL. Esters, higher alcohols, final pH value, attenuation, fermentable sugars and diacetyl concentration were analysed. Yakobson discovered that the influence of the pitching rate was strain dependent as both increased and decreased attenuation could be observed by increasing the pitching rate of all six strains. The production of 4-vinylguaiacol was the only significant change in aroma compounds that could be observed for all six strains by varying the pitching rate. As the pitching rate was increased, the overall production of 4-vinylguaiacol decreased. Following these findings, all other fermentations were conducted at a pitching rate of 12 × 10^6 cells/mL (Tables 11 and 12). All six strains were variable in their utilisation of the different wort sugars (Table 10). WY 5112 showed higher amounts of glucose and fructose in the beer than the starting wort. This may indicate that an external and internal α-glucosidase enzyme from *B. bruxellensis* broke down higher sugars to result in more free glucose and fructose. He reported further that WLP 653 partly fermented maltose but also left a significant amount of glucose. Strains CMY 001 and BSI-Drie showed the highest utilisation of all wort sugars.

In terms of the volatile compounds, it was found that most of the strains produced high amounts of ethyl caproate (sweet, fruity, pineapple) and ethyl caprylate (e.g. waxy, musty, sweet) (Tables 11 and 12). Ethyl acetate production varied from strain to strain while isoamyl acetate could not be detected at all. Yakobson *(89)* further described a low production of higher alcohols, which could be related to relatively low growth rates of the strains as well as to poor attenuation of some strains. At the end of his study the author judged *B. bruxellensis* to be a yeast capable of pure culture fermentation for the production of beers with special character.

Lentz et al. *(5)* fermented three worts (10, 12 and 14°P) with two strains of *B. bruxellensis* (Bc02, Bc07), which they isolated from fruits. Sugar assimilation tests were performed by API 20 (Analytical Profile Index) and strains tested for their ethanol tolerance. Beers were then analysed by trained panellists. Results showed that Bc02 fermented all three worts to an apparent attenuation of ~80%. However, the API 20 tests showed no assimilation of maltose (Table 13), whereas Bc07 showed only 20–25% of apparent attenuation by fermenting the 10 and 12°P wort. Both strains

Table 9. Results of the measurements of secondary metabolites formed during two fermentations at differing temperatures of one pure *B. bruxellensis (92)*

Fermentation temperature		25 °C	27 °C
Esters (mg/L)	Ethyl acetate	25	n.a.
	Ethyl-2-phenyl acetate	0.12	n.a.
	Ethyl isovalerate	0.32	n.a.
	Ethyl caproate	0.25	n.a.
	Ethyl caprylate	0.25	n.a.
	Ethyl caprate	0.17	n.a.
	Ethyl lactate	10	n.a.
	Diethylsuccinate	0.06	n.a.
	Ethylphenyl acetate	0.18	n.a.
	All esters	37.6	n.a.
Volatile fatty acids (mg/L)	Acetic acid	198	n.a.
	Propionic acid	0.09	n.a.
	Isobutyric acid	0.36	n.a.
	Butyric acid	0.02	0.23
	Isovaleric acid	3.9	14
	Valeric acid	0	n.a.
	Caproic acid	1	1.4
	Caprylic acid	3.1	2.7
	Capric acid	4.3	2.5
	Lauric acid	1.5	0.25
Non-volatile fatty acids (mg/L)	Oxalate	7.3	n.a.
	Citrate	103	n.a.
	Malate	84	n.a.
	Fumarate	0.3	n.a.
	Succinate	56	n.a.
	Lactate	184	n.a.
	Pyruvate	7.4	n.a.
	2-Ketoglutarate	1.3	n.a.
	Pelargonic acid	n.a.	0.22
	Palmitic acid	n.a.	0.09
Other acids (mg/L)	Phenylacetic acid	n.a.	7.5
Higher alcohols (mg/L)	Isobutanol	n.a.	0.99
	n-Butanol	n.a.	0.22
	i-Pentanol	n.a.	27
	Hexanol-1	n.a.	0.02
	Benzyl alcohol	n.a.	0.04
	2-Phenyl ethanol	n.a.	50
	Furfuryl alcohol	n.a.	2
	Methionol	n.a.	0.67
Phenols (mg/L)	Vanilin	n.a.	0.29
	4-Ethyl guaiacol	n.a.	2.6
	4-Ethylphenol	n.a.	0.82

showed moderate growth in MYPG media at ethanol concentrations of 12% v/v. The panellists evaluated both strains as unlikely to succeed as a brewing strain.

Pozippe et al. *(78)* used one *B. bruxellensis* strain (LTQB6) in wort (O.G. 16°P) and fermented it for 10 days at 28 °C. The ethanol content, volatile compounds and cell number were analysed. The beers that were produced had an ethanol concentration of about 4% (v/v) and showed comparatively small amounts of acetic acid (55.4 mg/L) and high amounts of acetaldehyde (26.3 mg/L). The isoamyl-alcohol content was reported to be 57.7 mg/L and that of higher alcohols to be 97.7 mg/L (Table 14). The authors suggested that the fermentation procedure should be adjusted (change of temperature) to lower the amount of off-flavours that the panellists described in the tastings. The type of off-flavours were not specified in the study.

Gamero et al. *(90)* used strain CBS 2796 to ferment commercial wort at 12 °C and reported a low quantity of isoamyl acetate (~1 mg/L), phenylethyl acetate (<1 mg/L) and ethyl caproate (~2 mg/L). They suggest that this yeast strain has a high potential for use in brewing and further research.

Preiss et al. *(99)* used eight different *B. bruxellensis* strains and fermented 70 mL of 12.5°P wort in triplicate for each temperature at 15 and 22.5 °C for 28 days. They reported a higher attenuation of the wort at 22.5 °C whereas the fermentation at 15 °C showed rather slow fermentation speed. Two of the strains displayed no fermentation ability. They analysed ethyl caproate, ethyl caprylate, ethyl decanoate, 4-vinylguaiacol, 4-ethylguaiacol and 4-ethylphenol, which are mostly responsible for the 'Brett-flavour'. Although the fermentation was described as poor at 15 °C, these flavour compounds were generally produced in almost equal amounts. In the case of ethyl caprylate and ethyl decanoate, some strains produced more at low temperatures and some produced more at high temperatures. The compound 4-ethylphenol was produced equally across the strains and at both temperatures in quantities of ~200 ppm.

Most of the studies reviewed above *(78,89,90)* suggested the use of *B. bruxellensis* as a pure brewing strain, except for Lentz et al. *(5)*. The formation of a large variety of esters such as ethyl acetate, ethyl caproate and ethyl caprylate in large quantities can produce beers with fruity and complex aromas *(78,91,99)*. Most authors reported very low quantities of isoamyl acetate, which could be explained by the ability of *B. bruxellensis* to break down this aroma compound, which is highly desirable in wheat beer *(95,98,99)*. The fermentation temperature seems to be highly variable as Gamero et al. *(90)* fermented at 12 °C, other authors fermented at ~20 °C, and Methner fermented at 25 and 27 °C, whilst all of them reported good flavours *(78,89,90,92)*. Preiss et al. *(99)* reported lower attenuations at lower temperatures but still high flavour formation, suggesting that the temperature had a very minor influence on the flavour. However, the fermentation time in most studies seems very long compared with fermentations conducted with the *Saccharomyces* brewing yeast. Only Pozippe et al. *(78)* fermented

Table 10. Different sugar contents of the final beers fermented with six different *B. bruxellensis* strains by Yakobson *(89)*

Fermentable sugars	Original wort	WY 5112	WLP 650	WLP 653	WY 5526	CMY 001	BSI-Drie
Glucose (g/L)	10.1	28.37	0	6.11	0	0	0
Fructose (g/L)	1.75	2.44	0	0.62	0	0	0
Sucrose (g/L)	5.65	0.53	0	2.41	0	0	0
Maltose (g/L)	72.78	34.45	54.77	51.15	47.86	27.03	5.75
Maltotriose (g/L)	18.39	18.79	15.89	15.95	16.38	6.16	8.62

Table 11. Summary of the results of fermentations performed in Yakobson's study with three different pitching rates and five different pH values *(89)*

Yeast strain	WY 5526						
Cell count (cells/mL × 10^6)/lactic acid (mg/L)	6/0	18/0	12/0	12/100	12/500	12/1000	12/3000
Apparent attenuation (%)	n.a.	n.a.	37.75	38.18	46.6	56.01	70.12
Final pH value	n.a.	n.a.	4.09	3.96	3.72	3.55	3.2
Acetaldehyde (mg/L)	1.03	1.24	1.05	n.a.	n.a.	n.a.	n.a.
Ethyl acetate (mg/L)	4.04	13.98	8.38	6.78	12.05	17.95	20.99
Ethyl lactate (mg/L)	0.84	0.71	0.75	1.48	3.9	7.56	35.99
Ethyl butyrate (mg/L)	0.02	0.05	0.05	n.a.	n.a.	n.a.	n.a.
Ethyl caproate (mg/L)	0.17	0.28	0.29	0.15	0.23	0.17	0.13
Ethyl caprylate (mg/L)	0.99	1.32	1.34	0.83	1.22	0.76	0.22
n-Propanol (mg/L)	1.88	3.88	3.01	n.a.	n.a.	n.a.	n.a.
Isobutanol (mg/L)	2	4.79	3.16	n.a.	n.a.	n.a.	n.a.
2-Methylbutanol (mg/L)	0.95	1.93	1.49	n.a.	n.a.	n.a.	n.a.
3-Methylbutanol (mg/L)	2.98	7.81	5.39	n.a.	n.a.	n.a.	n.a.
4-Vinylguaiacol (mg/L)	0.062	0.043	0.051	0.07	0.01	0	0
4-Vinylphenol (mg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diacetyl (mg/L)	0.048	0.06	0.051	n.a.	n.a.	n.a.	n.a.
2,3-Pentanedione (mg/L)	0.004	0.003	0.003	n.a.	n.a.	n.a.	n.a.
	CMY 001						
Cell count (cells/mL × 10^6)/lactic acid (mg/L)	6/0	18/0	12/0	12/100	12/500	12/1000	12/3000
Apparent attenuation (%)	47.85	56.53	53.89	58.35	60.5	71.14	87.27
Final pH value	n.a.	n.a.	4.06	3.92	3.76	3.58	3.25
Acetaldehyde (mg/L)	1.57	1.75	1.26	n.a.	n.a.	n.a.	n.a.
Ethyl acetate (mg/L)	12.98	22.85	16.76	18.88	24.59	33.77	40.65
Ethyl lactate (mg/L)	1.57	1.14	1.44	2.4	6	14.86	51.8
Ethyl butyrate (mg/L)	0.05	0.06	0.06	n.a.	n.a.	n.a.	n.a.
Ethyl caproate (mg/L)	0.32	0.33	0.39	0.44	0.29	0.25	0.11
Ethyl caprylate (mg/L)	2.79	2.77	3.35	1.93	1.45	0.82	0.52
n-Propanol (mg/L)	3.48	4.02	3.72	n.a.	n.a.	n.a.	n.a.
Isobutanol (mg/L)	6.83	6.35	7.04	n.a.	n.a.	n.a.	n.a.
2-Methylbutanol (mg/L)	2.69	2.48	2.67	n.a.	n.a.	n.a.	n.a.
3-Methylbutanol (mg/L)	5.96	7.02	6.71	n.a.	n.a.	n.a.	n.a.
4-Vinylguaiacol (mg/L)	0.046	0.026	0.039	0.09	0.09	0.07	0.07
4-Vinylphenol (mg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diacetyl (mg/L)	0.029	0.028	0.029	n.a.	n.a.	n.a.	n.a.
2,3-Pentanedione (mg/L)	0.004	0.004	0.004	n.a.	n.a.	n.a.	n.a.
	BSI-Drie						
Cell count (cells/mL × 10^6)/lactic acid (mg/L)	6/0	18/0	12/0	12/100	12/500	12/1000	12/3000
Apparent attenuation (%)	82.16	64.24	73.83	85.67	70.03	84.18	88.44
Final pH value	n.a.	n.a.	3.97	3.89	3.72	3.56	3.23
Acetaldehyde (mg/L)	1.98	1.85	1.98	n.a.	n.a.	n.a.	n.a.
Ethyl acetate (mg/L)	29.88	30.69	35.8	42.63	39.51	37.69	27.46
Ethyl lactate (mg/L)	1.94	1.18	1.29	2.91	9	17.51	70.26
Ethyl butyrate (mg/L)	0.08	0.06	0.08	n.a.	n.a.	n.a.	n.a.
Ethyl caproate (mg/L)	0.4	0.27	0.36	0.32	0.23	0.17	0.13
Ethyl caprylate (mg/L)	3.65	2.17	3	2	1.42	0.82	0.54
n-Propanol (mg/L)	6.62	4.97	6.56	n.a.	n.a.	n.a.	n.a.
Isobutanol (mg/L)	8.49	6.73	8	n.a.	n.a.	n.a.	n.a.
2-Methylbutanol (mg/L)	2.74	2.52	2.61	n.a.	n.a.	n.a.	n.a.
3-Methylbutanol (mg/L)	8.27	7.47	8.62	n.a.	n.a.	n.a.	n.a.
4-Vinylguaiacol (mg/L)	0.05	0.042	0.046	0.11	0.1	0.08	0.04
4-Vinylphenol (mg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Diacetyl (mg/L)	0.033	0.027	0.029	n.a.	n.a.	n.a.	n.a.
2,3-Pentanedione (mg/L)	0.005	0.004	0.004	n.a.	n.a.	n.a.	n.a.

n.a., Not analyzed; n.d., not detected.

Table 12. Summary of the results of fermentations performed in Yakobson's study with one pitching rate and five different pH values *(89)*

Yeast strain	WY5112				
Cell count (cells/mL × 10^6)/lactic acid (mg/L)	12/0	12/100	12/500	12/1000	12/3000
Apparent attenuation (%)	28.02	25.65	22.01	33.47	17.57
Final pH value	4.15	3.97	3.82	3.63	3.18
Acetaldehyde (mg/L)	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl acetate (mg/L)	1.88	2.03	2.92	1.6	1.56
Ethyl lactate (mg/L)	1.72	1.93	3.34	4.95	15.42
Ethyl butyrate (mg/L)	0	n.a.	n.a.	n.a.	n.a.
Ethyl caproate (mg/L)	0.11	0.07	0.08	0.08	0
Ethyl caprylate (mg/L)	0.72	0.3	0.44	0.13	0
n-Propanol (mg/L)	0.88	n.a.	n.a.	n.a.	n.a.
Isobutanol (mg/L)	2.46	n.a.	n.a.	n.a.	n.a.
2-Methylbutanol (mg/L)	1.31	n.a.	n.a.	n.a.	n.a.
3-Methylbutanol (mg/L)	2.15	n.a.	n.a.	n.a.	n.a.
4-Vinylguaiacol (mg/L)	0.04	0.06	0.05	0.04	0.03
4-Vinylphenol (mg/L)	n.d.	n.d.	n.d.	n.d.	n.d.
Diacetyl (mg/L)	0.056	n.a.	n.a.	n.a.	n.a.
2,3-Pentanedione (mg/L)	0.009	n.a.	n.a.	n.a.	n.a.
	WLP 650				
Cell count (cells/mL × 10^6)/lactic acid (mg/L)	12/0	12/100	12/500	12/1000	12/3000
Apparent attenuation (%)	26.49	27.37	35.61	36.69	57.47
Final pH value	4.18	4.16	3.9	3.62	3.29
Acetaldehyde (mg/L)	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl acetate (mg/L)	3.62	3.67	3.48	6.5	11.68
Ethyl lactate (mg/L)	0.18	1.58	4.36	7.62	34.65
Ethyl butyrate (mg/L)	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl caproate (mg/L)	0.16	0.16	0.12	0.16	0.22
Ethyl caprylate (mg/L)	1.65	1.39	0.77	1.07	1.2
n-Propanol (mg/L)	2.57	n.a.	n.a.	n.a.	n.a.
Isobutanol (mg/L)	3.83	n.a.	n.a.	n.a.	n.a.
2-Methylbutanol (mg/L)	5.22	n.a.	n.a.	n.a.	n.a.
3-Methylbutanol (mg/L)	5.13	n.a.	n.a.	n.a.	n.a.
4-Vinylguaiacol (mg/L)	0.05	0.1	0.09	0.08	0.06
4-Vinylphenol (mg/L)	n.d.	n.d.	n.d.	n.d.	n.d.
Diacetyl (mg/L)	0.024	n.a.	n.a.	n.a.	n.a.
2,3-Pentanedione (mg/L)	0.004	n.a.	n.a.	n.a.	n.a.
	WLP 653				
Cell count (cells/mL × 10^6)/lactic acid (mg/L)	12/0	12/100	12/500	12/1000	12/3000
Apparent attenuation (%)	50.45	61.03	39.02	54.76	55.28
Final pH value	4.02	3.93	3.68	3.59	3.29
Acetaldehyde (mg/L)	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl acetate (mg/L)	12.25	7.22	17.02	21.05	33.18
Ethyl lactate (mg/L)	1.61	2.65	4.41	10.51	33.33
Ethyl butyrate (mg/L)	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl caproate (mg/L)	0.25	0.24	0.17	0.11	0.09
Ethyl caprylate (mg/L)	4.13	2.5	1.89	1.67	0.96
n-Propanol (mg/L)	4.14	n.a.	n.a.	n.a.	n.a.
Isobutanol (mg/L)	2.32	n.a.	n.a.	n.a.	n.a.
2-Methylbutanol (mg/L)	1.09	n.a.	n.a.	n.a.	n.a.
3-Methylbutanol (mg/L)	3.82	n.a.	n.a.	n.a.	n.a.
4-Vinylguaiacol (mg/L)	0.02	0.08	0.06	0.05	0.05
4-Vinylphenol (mg/L)	n.d.	n.d.	n.d.	n.d.	n.d.
Diacetyl (mg/L)	0.22	n.a.	n.a.	n.a.	n.a.
2,3-Pentanedione (mg/L)	0.018	n.a.	n.a.	n.a.	n.a.

n.a., Not analyzed; n.d. not detected.

Table 13. Sugars assimilated by two *B. bruxellensis* strains tested for brewing ability by Lentz et al. *(5)*

Assimilated sugars	BC 02	BC 07
Glucose	+	+
Sucrose	–	–
Maltose	–	–

for 10 days and reported good flavour. Compared with the high OG of 16°P and a final ethanol content of 4% v/v it seems likely that the attenuation could have been higher if fermented for a longer time. Yakobson *(89)* found a significant change in 4-vinylguaiacol at varying pitching rates. When increasing the pitching rate, the overall production of 4-vinylguaiacol decreased. Overall it appears that the interest in brewing with pure *Brettanomyces bruxellensis* is not unfounded and that very complex and new beer types can be created depending on the strain used fermentation temperature and wort composition.

Candida shehatae. *Candida shehatae* has been investigated mostly for the fermentation of D-xylose and the production of ethanol. It has been reported to form ethanol in high yields compared with the fermented sugars *(100–102)*. Further studies with this species have reported that it is capable of bioreducing monoterpenes such as myrcene, which is present in beer wort coming from hops, into desirable flavour-active compounds *(103,104)*. This process has also been reported for other yeast strains by King and Dickinson *(55)*. It could be useful for developing more flavour-active hop compounds in beer and to contribute to an even larger variety in hop flavour for brewers. Further fermentation of sugar sources includes glucose, galactose, trehalose and maltose, which might indicate that this species could be used to ferment beer wort (Table 1).

Li et al. *(105)* used *C. shehatae* to ferment a wort (O.G. 9°P) in batches of 300 mL at 14 °C to produce a beer with a low alcohol concentration. The strain CICC 1766 (Chinese Industrial Culture Collection) of *C. shehatae* used was maltose negative in comparison with the type strain CBS 5813. After fermentation they verified that the strain did not ferment maltose. The ethanol content of the finished product did not exceed 0.5% (v/v) and could therefore be considered a non-alcoholic beer (for most countries). After the first trial they used 200 L of the same wort and fermented it with 1.5 L of pitching yeast which they propagated at 28 °C. Low ethanol contents ranging between 0.36 and 0.38% (v/v) were observed as well as a slightly higher pH value of 4.7 on average (compared with 4.6 before). Diacetyl levels were below the threshold of 0.1 mg/L in both trials (0.048 mg/L and 0.041 mg/L). The produced beers were analysed by panellists, who could not determine any wort-like flavour. However, the panellists could not detect a difference between the taste of the beer produced with *C. shehatae* and non-alcohol beers.

As only one study has been performed on producing beer with *C. shehatae*, the consideration of the ability to produce beer with this kind of yeast cannot be fully evaluated. The aroma of the produced beers should be analysed and more strains should be screened for more knowledge of the actual flavour compounds produced by this yeast.

Candida tropicalis. *Candida tropicalis* can be found in many different types of fermented indigenous foods and beverages. These products are primarily spontaneously fermented and *C. tropicalis* is one of the main organisms that occurs in these beverages along with lactic acid bacteria and other yeast strains *(106)*. *C. tropicalis*

Table 14. Volatile compounds measured in finished beer after fermentation with *B. bruxellensis* strain LTQB6 *(78)*

Volatile compounds	Acetaldehyde	Ethyl acetate	1-Propanol	Isobutanol	Isoamyl alcohol	Acetic acid	Furfural	Higher alcohols
LTQB6	26.3 mg/L	21.9 mg/L	21.7 mg/L	18.3 mg/L	57.7 mg/L	55.4 mg/L	7 mg/L	97.7 mg/L

Table 15. Summary of all trial fermentations performed with *S. ludwigii* with strain code, wort attributes, fermentation attributes and source of publication

Strain codes	Wort attributes			Fermentation parameters					Source
	pH	OG (°P)	IBU	Temp.(°C)	Pitching rate (cells/mL) × 10^6	Batch size (L)	Time	Storage	
S. ludwigii	NM	11.5	NM	20	NM	NM	120 h	NM	*(66)*
DSM 3447	4.87	6.5	NM	7	NM	0.3	235 h,	NM	*(118)*
				12			168 h		
DSM 3447	NM	NM	NM	4	10, 40	NM	48 h	NM	*(119)*
				12	10, 40				
				24	10, 40				
TUM SL 17	4.6	12.8	15	15	8	30	144 h	NM	*(111)*
	5.2	7	15	20	8	30	144 h		
DBVPG 3010	5.57	12	NM	20	NM	0.05	240 h	None	*(110)*
DBVPG 3304									
DBVPG 3398									
DBVPG 3931									
DBVPG 4116									
DBVPG 6721									

NM, Not Mentioned; OG, original gravity; IBU, international bitterness units.

is considered to be an (opportunistic) pathogen *(107)* and therefore this factor needs to be considered when producing beer. *C. tropicalis* strains are mostly able to ferment glucose, sucrose, and maltose as seen in Table 1.

N'Guessan et al. *(108)* used commercial brewery sorghum wort (O.G. 13°P) and pitched it with 1×10^6 cell/mL of a strain of *C. tropicalis*, which was isolated from a spontaneous fermentation of sorghum beer. Fermentation was carried out at 35 °C and observed for 12 h. The results suggested low growth rates during the first 4 h of fermentation. They also observed that the beer contained 20.5 g/L of lactic acid and had an ethanol concentration of 0.2% (v/v). No higher alcohols or acetaldehyde could be measured above 1 mg/L. However, the only volatile compound that was found in higher concentrations, although still much lower than the threshold (80 mg/L), was 2-butanone (5.52 mg/L).

Allouse-Boraud et al. *(109)* used strain F0–5 (*C. tropicalis*) from the culture collection of the University of Nangui Abrogoua to ferment a wort made from malt extract (12 g/L maltose, 2 g/L glucose). The wort was pitched with 1×10^6 cells/mL at 30 °C and samples were taken after 4, 8, 16 and 48 h. The initial pH value averaged about 5.8 and dropped to about 5.0 after 48 h. Low amounts of succinate (0.08 g/L) and lactate (0.15 g/L) were reported as well as an ethanol concentration of ~1.5% (v/v).

C. tropicalis does not produce many aroma compounds, as shown by the two studies of N'Guessan et al. *(108)* and Allouse-Boraud et al. *(109)*. The fermentation temperatures in these studies were high in comparison with all the other studies in this review and might be reconsidered. The actual pathogenicity of the strains should also be investigated before using them.

Saccharomycodes ludwigii. *Saccharomycodes ludwigii* has become a commonly used yeast for the production of low-alcohol or alcohol-free beers in Germany and Italy *(7,17,110,111)*. It is a good example of the novel use of non-*Saccharomyces* yeasts in the brewing industry. This morphologically lemon-shaped yeast was formerly considered to be a spoilage organism for wine. It has a high tolerance towards ethanol and SO_2, which gives it the ability to survive in most wines. It was first investigated and found to positively influence wine flavour – some of these flavours are important compounds that can occur in beer – and it produced high quantities of ethyl acetate and higher alcohols *(112)*. *S. ludwigii* was also investigated for its flavour-enhancing impact on spirit aroma where it was used to co-ferment grape mark. It produced high quantities of higher alcohols but did not show a significant influence on the overall aroma of the final product *(113)*. This species does usually not utilise maltose nor maltotriose in beer wort; it forms low concentrations of alcohol by only fermenting glucose, fructose and sucrose (Table 1) *(85,110,111,114)*. In 1929, Haehn and Glaubitz took out a patent for producing a beer with a low alcohol content fermented with *S. ludwigii (115)*. In 1990, Huige et al. *(116)* took out a similar patent for the process of preparing a non-alcoholic (<0.5 vol% alcohol) malt beverage that was also fermented by *S. ludwigii*.

Table 15 shows a summary of all the trials performed with pure *Saccharomycodes ludwigii* followed by an explanation of the process and the outcome of the different studies.

Narziß et al. *(66)* reported the slow attenuation of *S. ludwigii* of an 11.5°P wort at 20 °C. They also stated that wort acidification had a significant high impact on forming sensorial active by-products. The beer they produced had an ethanol concentration of 0.68% (v/v) so they suggested diluting the wort to 7.5°P to produce an ethanol concentration of 0.45% (v/v). The sensorial evaluation showed a masking of the typical wort-like flavour by the secondary metabolites that were generated (esters and higher alcohols, Table 16). However, a slightly wort-like flavour was constant.

In 2010 Sohrabvandi et al. *(117)* used several media containing different fermentable sugars and found that the *S. ludwigii* strain DSM 3447 had the highest growth rate and pH drop by a media containing fructose as a single fermentable sugar. Mohammadi et al. *(118)* used the same strain as Sohrabvandi et al. and immobilised it on delignificated brewer's spent grain. They fermented wort of 6.5°P at 7 and 12 °C and found that *S. ludwigii* could partly ferment maltose while being immobilised. They also suggested that temperature had a great influence on fermentation performance and reported that repitching five times had no influence on the fermentation rate of *S. ludwigii*.

Mortazavian et al. *(119)* fermented beer wort with *S. ludwigii* for 48 h at 4, 12 and 24 °C under aerobic conditions as well as under periodic aeration performed every 12 h. Growth of yeast cells was assessed, along with pH value and ethanol content, and a sensory evaluation was conducted. The resulting beers showed positive acceptance by all the panellists. However, the beer produced at 24 °C had a sour taste owing to the production of lactic acid.

Meier-Dörnberg et al. *(111)* used *S. ludwigii* TUM SL 17 at a pitching rate of 8×10^6 cells/mL in two worts with different original gravities of 7 and 12.8°P. They adjusted the pH value of the 12.8°P wort to 4.6 as suggested by Narziß et al. *(66)*. The fermentation had a slow fermentation rate, low ethanol concentration of 0.48% (v/v) and a high acceptance as a well-flavoured beer by the panellists judging the beer. However, they found that a 7°P wort (pH 5.2) fermented with TUM SL 17 at 15 °C showed higher quantities of amyl alcohols and produced greater quantities of higher alcohols compared with the average of 20 German alcohol-free wheat beers. They also reported low concentrations of ethyl acetate (0.65 mg/L), isoamyl acetate (0.1 mg/L), and 4-vinylguaiacol (0.1 mg/L). The overall flavour of the beers was described as being honey-like.

De Francesco et al. *(110,120)* screened six different *S. ludwigii* strains, most of them isolated from grape must, for low-alcohol beer production. They fermented a 12°P wort at 20 °C for 10 days, which they had produced by high-temperature mashing with the aim of high amounts of non-fermentable sugars being present in the wort. One strain (DBVPG 3100) displayed the lowest ethanol level produced at 0.51% v/v but the highest content of total

Table 16. Volatile compounds measured in finished beer after fermentation with an *S. ludwigii* strain by Narziß et al. *(66)*

Strain used *S. ludwigii*	
Secondary metabolites	(mg/L)
Ethyl acetate	1.8
Ethyl hexanoate	0.01
Ethyl octanoate	0.01
Total esters	1.82
n-Propanol	2.0
Isobutanol	7.50
3-Methyl-2-butanol	15
2-Methyl-1-butanol	7.50
Furfuryl alcohol	4.40
2-Phenylethanol	8.90
Total higher alcohols	45.30
Diacetyl	0.14
2,3-Pentanedione	0.001

produced esters of 15 mg/L (Table 17). It also formed the highest level of off-flavours that did not exceed the threshold level, which were therefore undetected by the panellists. All other strains formed ethanol at concentrations between 0.7 and 1.4% v/v, which legally cannot be described as alcohol-free beer in most countries.

As *S. ludwigii* is already in use in some German as well as Italian and Czech breweries, it can be viewed as a good example of a non-*Saccharomyces* strain being implemented in the brewing industry. The low-alcohol or alcohol-free beers that are produced have an ethanol concentration of about 0.5% (v/v) and display good flavour *(111)*. As shown by De Francesco et al. *(110)*, the production of esters as well as the ethanol content of the final beer is strain dependent. In terms of the wort-like off flavour that is often described by panellists when judging alcohol-free beer, it appears that this species is able to cover wort-like off flavour by producing masking flavour compounds *(66,111,119)*. Most of the authors reported good flavours when fermenting at temperatures of around 20 °C and a low original gravity around 7°P *(66,111)*.

Zygosaccharomyces rouxii. *Zygosaccharomyces rouxii* has been used mainly by Iranian, Dutch, and Italian scientists to produce a low-alcohol or alcohol-free beer *(63,90,118,120)*. Its high salt and sugar tolerance means that it is also known to the food industry as a spoilage yeast. Owing its high osmotic tolerance, it was tested as a baking yeast but did not give a good aroma, texture or taste characteristic in the final breads *(121)*. The high osmotic tolerance could potentially be used in high-gravity brewing since some strains have shown to ferment all wort sugars (Table 1) *(73,85)*. However, it produces high quantities of ethyl acetate, amyl alcohols and isoamyl alcohols in soy sauce, which are well-known flavour compounds to brewers *(122)*. Furthermore, Lee et al. *(123)* studied the volatile profile of soy sauce fermented with *Z. rouxii* and showed that this species produces many of the flavour-active compounds such as esters and higher alcohols that are also desired in beer. It also showed a low production of undesired acids.

Table 18 gives a summary of all the trials conducted with pure *Zygosaccharomyces rouxii* followed by an explanation of the process and the outcome of the different studies.

Two *Z. rouxii* strains (DSM 2535 and 2531) were used along with two other species (*S. cerevisiae* and *S. ludwigii*) to ferment different media containing varying sugar sources by Sohrabvandi et al. *(63)*. The aim was to find suitable yeast strains for producing non-alcoholic beer. The fermentation was performed for 48 h at 24 °C with periodic aeration. Cell growth, ethanol production, pH drop and change in attenuation were measured. *Z. rouxii* showed maximum growth in the media containing fructose as well as glucose, which also gave the highest pH drop (pH Δ0.9). They found strain DSM 2531 to be suitable for producing non-alcoholic beer owing to its inability to ferment maltose or maltotriose.

Mohammadi et al. *(118)* used strain *Z. rouxii* DSM 2531 and immobilised it on brewer's spent grain. They fermented beer wort at 12 °C with original gravities of 6.5 and 9°P and at pH values of 4.87 and 7. Fermentation continued until a final density of 1.8–4.4°P was reached. Repitching showed that *Z. rouxii* increased its fermentation speed after the fourth batch but then remained constant for three subsequent batches. It produced an alcohol concentration of 4.1% (v/v) at 7 °C and 9°P after 235 h and 4.8% v/v with the same density at 12 °C after 168 h of fermentation.

Gamero et al. *(90)* screened 7000 yeast strains for diversification of flavour in beer and found *Z. rouxii* to have interesting characteristics for brewing along with 11 other strains of different species. For each strain they fermented 200 mL of beer wort and 200 mL of an enzyme-treated wort that was high in glucose, and analysed the flavour, ethanol content and sugar uptake. *Z. rouxii* displayed a very low ester production of ~2 mg/L but a fast fermentation speed.

Mortazavian et al. *(119)* fermented beer wort (6°P) with two different strains of *Z. rouxii* for 48 h at 4, 12 and 24 °C under aerobic conditions as well as under periodic aeration performed every 12 h. The growth of yeast cells was assessed, and the pH value and ethanol content were measured. The ethanol content in the final beers varied from 0.05% v/v (fermented at 4 °C) to 0.4% v/v (fermented at 24 °C). The authors stated that the beers produced

Table 17. Volatile compounds measured in finished beers after fermentation with six different *S. ludwigii* strains *(110)*

	Strains used					
Secondary metabolites	DBVPG 3010 (mg/L)	DBVPG 3054 (mg/L)	DBVPG 3304 (mg/L)	DBVPG 3398 (mg/L)	DBVPG 3931 (mg/L)	DBVPG 4116 (mg/L)
Ethyl acetate	14.86	2.02	1.52	1.17	2.31	4.11
Isoamyl acetate	0.022	0.008	0.009	0.011	0.008	0.010
Ethyl hexanoate	0.014	0.016	0.015	0.016	0.017	0.02
Ethyl octanoate	0.011	0.013	0.012	0.011	0.012	0.010
Total esters	14.91	2.06	1.56	1.21	2.358	4.15
n-Propanol	3.25	3.38	3.26	3.13	5.57	5.44
Isobutanol	5.27	6.55	10.76	8.78	15.31	12.72
3-Methyl-2-butanol	14.4	14.14	18.78	16.00	16.82	28.92
2-Methyl-1-butanol	4.48	3.72	4.81	4.37	6.95	6.98
Furfuryl alcohol	1.44	1.49	1.51	1.20	2.32	2.55
2-Phenylethanol	14.47	13.71	14.73	14.18	15.36	20.01
Total higher alcohols	43.31	42.99	53.85	47.66	62.34	76.62
Acetaldehyde	4.42	3.36	2.79	1.85	2.02	2.74
Diacetyl	0.015	0.0055	0.006	0.006	0.010	0.005
2,3-Pentanedione	0.002	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected.

Table 18. Summary of all trial fermentations conducted with *Z. rouxii* with strain code, wort attributes, fermentation attributes and source of publication

Strain codes	Wort attributes			Fermentation parameters					Source
	pH	OG (°P)	IBU	Temp.(°C)	Pitching rate (cells/mL) × 10^6	Batch size (L)	Time	Storage	
DSM 2531	4.87	6.5 9	NM	7, 12	NM	NM	235 h, 168 h	NM	*(118)*
CBS 9716	NM	NM	NM	12	NM	0.2	NM	None	*(90)*
DSMZ 70535	NM	6	NM	4 12	10, 40	NM	48 h	NM	*(119)*
DSMZ 70531				24					
DBVPG 4084 DBVPG 6187 DBVPG 6424 DBPVG 6463 DBPVG 6921	5.57	12	NM	20	NM	0.05	240 h	None	*(110)*

NM, Not Mentioned; OG, original gravity; IBU, international bitterness units.

had a low acceptance along the panellists owing to the low degree of fermentation by-products.

De Francesco et al. *(110)* described *Z. rouxii* as a yeast that was capable of fermenting beer wort to a drinkable low-alcohol or alcohol-free beer. They took five different strains from Iraq (DBVPG 4084), Italy (DBVPG 6187), the Netherlands (DBVPG 6424), the USA (DBVPG 6463) and Canada (DBVPG 6921) and fermented a special wort with a low quantity of mono- and disaccharides having 12°P and a pH value of 5.57. They fermented 50 mL of the special wort at 20 °C and measured ethanol content, higher alcohols, esters, aldehydes and vicinal diketones. Ethanol contents varied from 0.9 to 3.32% v/v while high quantities of higher alcohols (92–196 mg/L) were produced. Among the five strains, the total esters ranged from 4.42 and 71.15 mg/L. The total amount of acetaldehyde also varied, ranging from 5.5 to 8.1 mg/L. All of the strains produced diacetyl above the threshold of 0.1 whilst DBVPG 6187 produced the highest value at 0.85 mg/L (Table 19) *(110,120)*.

The variance in ethanol production, flavour and off-flavour formation in this species seems very diverse. Some authors such as Mortazavian et al. *(119)* report very low ethanol contents, whereas Sohrabvandi et al. *(63)* found *Z. rouxii* to be unsuitable for alcohol-free beer production owing to the high alcohol concentrations in the final beers *(63,119)*. As De Francesco et al. *(110)* found a high quantity of esters produced by some of the investigated strains, they also reported high quantities of diacetyl and 2,3-pentanedione that were much higher than the typical taste thresholds *(22,110)*. However, none of the authors reported any maturation phase. In this case a diacetyl reduction following fermentation could have led to less diacetyl *(30)*. High production of isoamyl acetate could not be observed by any of the authors *(90,110,120)*. No trials have been performed so far on the production of average original gravity beers (e.g. 12°P) with this species. In terms of the high potential of flavour forming reported when using this yeast and the trials performed by De Francesco et al. *(110)*, who reported high

Table 19. Volatile compounds measured in finished beers after fermentation with five different *Z. rouxii* strains *(110)*

Secondary metabolites	Strains used				
	DBVPG 4084 (mg/L)	DBVPG 6187 (mg/L)	DBVPG 6424 (mg/L)	DBVPG 6463 (mg/L)	DBVPG 6921 (mg/L)
Ethyl acetate	4.38	33.68	7.35	70.86	2.12
Isoamyl acetate	0.013	0.069	0.02	0.21	0.008
Ethyl hexanoate	0.019	0.017	0.020	0.045	0.017
Ethyl octanoate	0.014	0.013	0.020	0.020	0.011
Total esters	4.42	33.78	7.41	71.15	2.16
n-Propanol	7.73	9.51	33.5	15.87	5.64
Isobutanol	37.76	28.67	68.89	45.91	14.59
3-Methyl-2-butanol	62.87	29.63	45.84	75.82	16.42
2-Methyl-1-butanol	8.72	11.50	19.15	20.38	6.76
Furfuryl alcohol	3.46	2.70	2.83	4.26	2.89
2-Phenylethanol	24.16	10.08	9.81	34.54	15.51
Total higher alcohols	146.69	92.07	180.05	196.77	61.80
Acetaldehyde	5.57	7.63	7.37	7.55	8.15
Diacetyl	0.28	0.85	0.23	0.25	0.66
2,3-Pentanedione	0.00001	0.00062	0.0028	0.0257	0.0667

quantities of esters and ethanol concentrations of 3.32% v/v, this has the potential to produce drinkable beers.

Pichia kluyveri. *Pichia kluyveri* has recently been discovered for producing low-alcohol or alcohol-free beers owing to its limited ability to ferment glucose whilst significantly changing hop compounds into positive flavour compounds (Table 1) *(64,124)*. It has also been isolated from various wine fermentations around the world *(125–127)*. *P. kluyveri* can produce noticeable amounts of volatile thiols such as 3-mercaptohexyl acetate (passion fruit aroma) and 3-mercaptohexan-1-ol (grapefruit aroma) in Sauvignon Blanc *(126)*. It was further reported that the fermentation speed of the species in tequila fermentation was very high compared with commercially used *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* strains. It also produced average levels of esters and high quantities of higher alcohols *(128)*. The flavour forming and fast fermentation of this yeast might indicate a brewing potential for novel alcohol-free beer *(64,126)*.

Saerens and Swiegers *(124)* suggested *P. kluyveri* for producing low-alcohol or alcohol-free beer because of their recent findings. They fermented batches of 1000 L of beer wort with an original gravity of 8.3°P with two different *P. kluyveri* strains (strains A and B, and inoculated 5×10^5 cells/mL) at 20 °C for 3 weeks to produce an alcohol-free beer. The worts used were unhopped as well as hopped with extract. To produce a low-alcohol beer they used 1500 L of the same wort but added different hops (Tettnang and Amarillo) and fermented these for 3 weeks at 21 °C only with strain A. Halfway through the fermentation they added Tettnang and Amarillo for dry hopping. Following fermentation they cooled the samples down to 4 °C. Beers were then analysed for acetate esters, ethyl esters and higher alcohols as well as ethanol content. The alcohol-free beer fermented with *P. kluyveri* strain A had an ethanol concentration of 0.1% v/v, isoamyl acetate quantity of 1.96 mg/L and isoamyl alcohol content of 2.00 mg/L. Strain B produced 0.2% v/v ethanol with the same level of isoamyl alcohols but 4.94 mg/L of isoamyl acetate. The low-alcohol beer had 0.7% v/v of ethanol, 2.5 mg/L isoamyl acetate and 1.8 mg/L isoamyl alcohols. Owing to these attributes Saerens and Swiegers suggest that *P. kluyveri* is a yeast that is ideally suited to producing alcohol-free and low-alcohol beers *(64)*. Saerens and Swiegers *(124)* also used *P. kluyveri* to ferment beer wort with an original gravity of 10°P which was divided into four batches with each being boiled with a different hop variety (Nelson Sauvin, Amarillo, Saaz and Cascade). Four 1 L batches of each wort were used with two being fermented at 20 °C and two at 22 °C. At each temperature *P. kluyveri* was inoculated to one batch and compared with a control fermentation with one *S. cerevisiae* with both inoculated at 5×10^5 cells/mL. After 2 days the *S. cerevisiae* strain was added to the samples fermented with *P. kluyveri.* After a total of 5 and 13 days samples were taken and analysed for flavour and ethanol concentration. Most esters were found to increase in the pre-fermented *P. kluyveri* wort and they saw increases in isoamyl- and isobutyl acetate. It was also reported that acetaldehyde and isoamyl acetate content increased by doubling the concentration of the control *S. cerevisiae* strain in the fermentations with Amarillo and Cascade. Furthermore, a variation in all the yeast-derived flavour compounds was due to the influence of hop variety *(124)*.

As no further investigations have been published using this yeast species to date, an evaluation of the brewing potential cannot be commented on despite the extensive analyses described above. Commercially the use of this strain has not been implemented.

Conclusion and perspectives

The studies discussed above show that some non-*Saccharomyces* yeasts are capable of fermenting beer wort into highly drinkable beers with varying flavours and alcohol content. They also demonstrate a large variance from strain to strain within species as well as among species with respect to sugar utilisation, degree and rate of fermentation, and flavour production. Most of the trials are performed on a laboratory scale and have not yet been scaled to an industrial level, which would give a more accurate assessment of their brewing ability. However, most of the trials were meant to be screenings for strains that have the potential for commercial production. The trials also highlight that the strains currently used in breweries are just a small sample of the diversity of yeasts that have potential for brewing among non-*Saccharomyces* yeasts. Characterising more unconventional yeast species for their brewing ability could offer brewers new strains to develop different styles of beer. By enriching the variety of brewing yeasts available, we will also have to disenthrall ourselves from the usual beer tastes and provide opportunities for developing new beers. The studies discussed in this review clearly show that even closely related strains can perform quite differently and the possibilities of finding new strains is limitless. However, one has to keep in mind that not all yeasts are harmless and there are (opportunistic) pathogens with the ability to ferment such as *C. tropicalis*. Overall, there is a severe lack of knowledge about non-*Saccharomyces* yeasts for use in industry. One should always be aware of the biosafety and approval by the European Food Safety Authority or the US Food Drug Administration *(129,130)*. If the information given by these authorities is taken into account, there is a great potential among the non-*Saccharomyces* strains for the discovery of new brewing strains.

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Part 2	2.3 Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as a model

When searching for new yeast strains that might be applicable to the fermentation of wort to beer the strains need to be selected in advance. To predict if a yeast will ferment an all-malt wort into a respectable beer, a variety of phenotypic tests can be applied. The screening developed in this publication describes these tests. Sugar and amino acid utilization, growth in the presence of hop compounds, ethanol resistance, and phenolic off-flavor (POF) tests were conducted to estimate the behavior of the applied yeast strains in a beer fermentation. Ten strains of *Torulaspora delbrueckii* from different habitats were taken through this screening to test the screening itself and then find a strain capable of fermenting an all-malt wort.

One strain (T9) was found that could utilize all wort sugars. No other strain could utilize maltose or maltotriose. All strains were able to tolerate 5 % v/v ethanol and up to 90 IBU and did not produce any POF. The cell growth as well as flocculation behavior was investigated before starting fermentation in triplicates. The fermentation temperature was set to 27 °C, the pitching rate was adjusted to 30 $*10^6$ cells/mL and the wort used was diluted from one batch of wort extract to ensure standardized conditions. High cell counts could be achieved with viabilities of 98.8-95.3 %. The fermentation behavior of all the applied strains showed the predicted outcome as only one strain was capable of completely fermenting the wort into a respectable beer (approx. 4 % v/v alcohol). All yeast strains were able to lower the pH of the final product to about 4.2. Trained panelists judged the produced beers as having fruity, floral and wort-like attributes. Beer fermented with T9 was judged to be the highest for fruity and floral and lowest in wort-like. T13 and T17 were also judged high in fruity and further suggested for low-alcohol production. The screening was therefore found to be applicable for the field of use and T9 was suggested for further research.

Authors/Authorship contribution:

Michel M.: Literature search, writing, data creation, study conception and design; **Kopecká J.**: Data analysis and interpretation (Real-time-PCR, Fingerprint system); **Meier-Dörnberg T.**: Creation of the research plan (fermentation); **Zarnkow M.**: support in the statistical analysis of data; **Jacob F.**: Supervised the project; **Hutzler M.**: Creation of the research plan, critical content review

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Research Article

Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model

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Abstract

This study describes a screening system for future brewing yeasts focusing on non-*Saccharomyces* yeasts. The aim was to find new yeast strains that can ferment beer wort into a respectable beer. Ten *Torulaspora delbrueckii* strains were put through the screening system, which included sugar utilization tests, hop resistance tests, ethanol resistance tests, polymerase chain reaction fingerprinting, propagation tests, amino acid catabolism and anabolism, phenolic off-flavour tests and trial fermentations. Trial fermentations were analysed for extract reduction, pH drop, yeast concentration in bulk fluid and fermentation by-products. All investigated strains were able to partly ferment wort sugars and showed high tolerance to hop compounds and ethanol. One of the investigated yeast strains fermented all the wort sugars and produced a respectable fruity flavour and a beer of average ethanol content with a high volatile flavour compound concentration. Two other strains could possibly be used for pre-fermentation as a bio-flavouring agent for beers that have been post-fermented by *Saccharomyces* strains as a consequence of their low sugar utilization but good flavour-forming properties. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: **non-Saccharomyces; Torulaspora delbrueckii; beer; screening; yeast**

Introduction

Saccharomyces cerevisiae is the main yeast species used in brewing (Lodolo *et al.*, 2008). Yeast is involved in most aroma-forming processes during beer fermentation, transforming wort ingredients into alcohol, and aroma compounds such as higher alcohols, esters and carbonyl compounds (Pires *et al.*, 2014). Common yeast species for producing various types of beer are described by Hutzler *et al.* (2015).

Recently, the traditional beverage beer has lost consumers to other innovative beverages. Brewers have tried to counteract this negative trend by expanding the hop varieties (De Keukeleire *et al.*, 2010), using special malts, going into craft brewing or creating new beer-blended beverages (Tremblay *et al.*, 2005; Vanderhaegen *et al.*, 2003; Statistisches Bundesamt 26.03.2015). Since one of the greatest changes in beer aroma can be achieved by using different yeast strains, the time has come to start searching for new yeasts besides the conventional strains of *Saccharomyces*. Non-*Saccharomyces* yeasts are mostly known as spoilage yeasts for beer or other beverages but they can actually form a diversity of flavours which might just fit beer perfectly (Pires *et al.*, 2014; Verstrepen *et al.*, 2003a). In addition to new aromas and flavours, there may be further benefits of using non-*Saccharomyces* yeasts for fermentation, such as a higher glycerine content for more mouthfeel (Andorrà *et al.*, 2010; Rantsiou

et al., 2012; Tofalo *et al.*, 2012). Another example shows that some of the non-*Saccharomyces* strains have a high content of enzymes that can transform monoterpenes. Monoterpenes are the main contributors to hop flavour, and examples of these substances are nerol, linalool and limonene (Wriessnegger and Pichler, 2013; Inui *et al.*, 2013; Takoi *et al.*, 2010). King investigated the non-*Saccharomyces* yeast *Torulaspora delbrueckii*, which was able to metabolize nerol to increase the amount of linalool. Having a higher content of linalool noticeably changed the hop flavour of beer (King and Dickinson, 2000). There is little research on the capabilities of non-*Saccharomyces* in brewing. Changing the yeast is one of the easiest adjustments for the average brewery to make because they usually already have equipment such as a propagation and fermentation vessel, and the only change they may need to make is the temperature during propagation, fermentation and maturation, though the risk of cross-contamination must be taken into account.

To find new yeast strains a screening system was developed to determine the ability of non-*Saccharomyces* yeasts to ferment beer wort to a new flavoured beer. When screening brewing yeasts, several key attributes need to be tested. The first attribute is the ability to utilize the wort sugars, as for most German beers the average composition is about 8.5% glucose and fructose, 4% sucrose, 42% maltose and 10.5% maltotriose (Narziß and Back, 1999; Narziß *et al.*, 2012). Another attribute is the utilization of amino acids, which varies like the sugar utilization from species to species and also from strain to strain (Procopio *et al.*, 2014; Andorrà *et al.*, 2010). Furthermore, the yeast should be able to grow in the presence of hop compounds. This was studied by Hazelwood in 2010 for the *Saccharomyces* species but was not investigated for non-*Saccharomyces* (Hazelwood *et al.*, 2010). Another important attribute for the production of beer is ethanol tolerance and the ability to produce alcohol (Lam *et al.*, 2014).

After finishing these main tests, the next aim is to propagate the strains. The main goal of propagation is to obtain a large quantity of high-quality biomass, meaning a high vitality and viability of the yeast cells (Hutzler *et al.*, 2015). Different parameters that are involved in this step include the assimilation temperature, aeration of the wort, composition of the wort with regard to sugars and amino acids, and the assimilation time (Wackerbauer *et al.*, 2002).

Some non-*Saccharomyces* yeasts can produce phenolic off-flavours, which are mostly unwanted in beer and wine (Shinohara *et al.*, 2000; Müller-Auffermann *et al.*, 2013; Scholtes *et al.*, 2014). Phenolic off-flavours are produced by decarboxylation of the acids that are present in beer wort, such as ferulic acid, coumaric acid and cinnamic acid. Ferulic acid is decarboxylated to 4-vinylguaiacol, which is one of the main flavour components in German wheat beer and is described as having a clove-like flavour (Coghe *et al.*, 2004). Besides the wheat beer style this flavour is mostly unwanted. Coumaric acid is decarboxylated to 4-vinylphenol, which is also identified as a solvent-like flavour, and cinnamic acid is decarboxylated to 4-vinylbenzol, which has a Styrofoam-like flavour (Scholtes *et al.*, 2014). So-called POF (phenolic off-flavour) tests are performed as part of screening.

After passing these screening steps, trial fermentations in 2-litre vessels are performed. The change in extract and pH value is examined daily. The finished beers are evaluated by a sensory panel. The alcohol content, fermentation by-products and a variety of flavour-active esters are also analysed.

To ensure the purity of all the strains and for further quality control at the brewery, all strains are examined by real-time polymerase chain reaction (PCR) and two fingerprint systems are applied for every strain.

The first yeast species that was selected for screening was *Torulaspora delbrueckii*. *Torulaspora delbrueckii* is well known from its use in the wine industry, where it is used to produce a fruitier flavour in wine (Albertin *et al.*, 2014). Recent studies show that *T. delbrueckii* (anamorph: *Candida colliculos*a) was domesticated by humans as far back as 4000 years ago (Albertin *et al.*, 2014). In 2003 it became the first commercially used non-*Saccharomyes* yeast sold for winemaking (Jolly *et al.*, 2014; Kurtzman *et al.*, 2011; Tataridis *et al.*, 2013). For this purpose it is distributed in yeast blends for high sugar mostly owing to its positive impact on flavour and its high sugar tolerance (Jolly *et al.*, 2014; Alves-Araújo *et al.*, 2004; Azzolini *et al.*, 2012). It has been reported that *T. delbrueckii* strains can be found in a large variety of habitats such as fruits, malt, soil and many more (Kurtzman *et al.*, 2011). Ten strains of *T. delbrueckii* were gathered from different habitats to obtain a

wide variety of strains with different flavour-forming abilities. One of the investigated yeast strains is already being used in brewing as pre-fermentation of wheat beer (Hutzler *et al.*, 2015).

The following section will deal with the structure of the screening system followed, by an overview of the results and a discussion of the screening itself, as well as the screening results.

Materials and methods

Yeast strains

Table 1 lists the yeast strains that were used in this study. Strains were grown on wort agar slopes for 72 h at 28 °C and stored in a sterile environment at 2–4 °C. The strains were subculture at intervals of 1 month. The strains were chosen from different culture collections and were marked with their official abbreviations.

Biochemical analysis

Substrate utilization tests API ID 32c (analytical profile index; BioMérieux. France) were used to analyse the biochemical spectrum of all 10 *T. delbrueckii* strains. Strains were taken from wort agar slopes and transferred to wort agar plates. Agar plates were inoculated for 2 days at 28 °C as suggested by the manufacturer. Identical colonies were picked from the plate and transferred to a 2 ml suspension medium, included in the API kit, until the turbidity equalled a 2 McFarland standard. 250 µl of the 2 ml inoculated suspension was transferred to a 7 ml API C medium. From this medium 135 µl was transferred to each of the 32 wells containing different substrates. Table 2 shows all 32 substrates and their quantity. After inoculating all the wells, API ID 32c plates were incubated for 2 days at 28 °C. The samples were evaluated visually by turbidity of the wells.

DNA extraction

Yeast DNA was isolated using the InstaGene™ Matrix (Bio-Rad. Munich. Germany). This was achieved by taking one pure culture of the investigated yeast strain from wort agar slope using an inoculation loop. The culture was transferred to a 1.5 ml tube and mixed with an aliquot of 200 µl InstaGene™ Matrix solution. Samples were vortexed for 10 s and incubated at 56 °C in a Thermomix 5436 (Eppendorf, Hamburg, Germany). Hereafter the sample was vortexed for 10 s and incubated at 96 °C for a further 8 min. After incubation, the sample was centrifuged at 12 000 × *g* for another 2 min and 100 µl of the DNA-containing sample was transferred to a new 1.5 ml tube. The described protocol was modified for yeast DNA extraction according to Hutzler (2009, 2010).

Table 1. Investigated yeast strains and their culture collection number or signature and origin

Designation	Species	Strain number/signature	Origin
T6	*T. del.*	RIBM[a] TdA	Wine
T9	*T. del*	DSM[b] 70504	Sorghum brandy
T10	*T. del*	CBS[c] 1146[T]	Unknown
T11	*T. del*	TUM[d] 214	Bottle (Pils beer, trace contamination, no beer spoilage observed)
T13	*T. del*	TUM[d] TD1	Wheat beer (starter culture)
T15	*T. del*	TUM[d] 138	Cheese brine
T17	*T. del*	WYSC/G[e] 1350	Unknown
T18	*T. del*	CBS[c] 4510	Unknown
T19	*T. del*	DSM[b] 70607	Unknown
T20	*T. del*	CBS[c] 817	Unknown
TUM 68	*S. cer*	TUM[d] 68	Top-fermenting yeast
TUM 34/70	*S. past.*	TUM[d] 34/70	Bottom-fermenting yeast

[a]RIBM collection – Research Institute of Brewing and Malting, Department of Microbiology, Prague, Czech Republic.
[b]DSM, Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany.
[c]CBS, Centrallbureau voor Schimmelcultures, Utrecht, Netherlands.
[d]TUM = Research Centre Weihenstephan for Brewing and Food Quality. TU München. Freising. Germany.
[e]WYSC/G, Weihenstephan Culture Collection of Yeast and Mould Strains, glycerol-stock, TU München, Freising, Germany.

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Table 2. Substrates used in API ID 32c

Test	Substrate	Quantity (mg/cup)	Test	Substrate	Quantity (mg/cup)
GAL	D-Galactose	0.70	SOR	D-Sorbitol	2.72
ACT	Cycloheximide (actidione)	0.014	XYL	D-Xylose	0.70
SUC	D-Sucrose	0.66	RIB	D-Ribose	0.70
NAG	*N*-Acetylglucosamine	0.64	GLY	Glycerol	0.82
LAT	Lactic acid	0.64	RHA	L-Rhamnose	0.68
ARA	L-Arabinose	0.70	PLE	Palatinose	0.66
CEL	D-Cellobiose	0.66	ERY	Erythritol	1.44
RAF	D-Raffinose	2.34	MEL	D-Melibiose	0.66
MAL	D-Maltose	0.70	GRT	Sodium glucoronate	0.76
TRE	D-Trehalose	0.66	MLZ	D-Melezitose	0.66
2KG	Potassium 2-ketogluconate	1.09	GNT	Potassium gluconate	0.92
MDG	Methyl-αD-glucopyranoside	1.92	LVT	Levulinic acid	0.48
MAN	D-Mannitol	0.68	GLU	D-Glucose	0.78
LAC	D-Lactose	0.70	SBE	L-Sorbose	0.70
INO	Inositol	0.70	GLN	Glucosamine	0.68
0	No substrate	—	ESC	Esculin	0.28

Table 3. Primers, probes and IAC135 components (internal amplification control) used for quantification of genomic DNA from the target microorganisms according to Hutzler (2009; Müller-Auffermann *et al.*, 2013)

Target yeast		Primer/probe sequence (5′–3′)	Reference
T. del	Td-f	AGATACGTCTTGTGCGTGCTTC	Hutzler (2009)
	Td-r	GCATTTCGCTGCGTTCTT	
	Y58	AACGGATCTCTTGGTTCTCGCATCGAT	
IAC135 components	135-f	TGGATAGATTCGATGACCCTAGAAC	Müller-Auffermann *et al.* (2013)
	135-r	TGAGTCCATTTTCGCAGATAACTT	
	135-S (HEX)	TGGGAGGATGCATTAGGAGCATTGTAAGAGAG	
	135 target DNA	TGCTAGAGAATGGATAGATTCGATGACCCTAG AACTAGTGGGAGGATGCATTAGGAGCATTGTA AGAGAGTCGGAAGTTA	
	135-rev target DNA	TGCGACACCTTGGGCGACCGTCAATAGGCCA CTCGAATGAGTCCATTTTCGCAGATAACTTCC GACTCTCTTACAATGCT	

Real-time PCR

Species classifications were verified using real-time PCR (Light Cycler 480 II, Roche, Germany). The primers and probe sequence Td-r, Td-f and Y58 were used according to Hutzler (2009, 2010). Typical real-time PCR was performed with 10 µl 2× Master Mix (Light Cycler 480 Probe Master. Roche. Germany), 1.4 µl PCR water, 0.8 µl of each primer (Biomers, Munich, Germany), 0.4 µl probe, 0.5 µl IAC135-f, 0.5 µl IAC135-r, 0.4 µl IAC135-S (HEX), 0.1 µl IAC135 ($1{:}10^{10}$), 0.1 µl IAC135 rev ($1{:}10^{10}$) and 5 µl template DNA with a total reaction volume of 20 µl. Real-time PCR parameters were: (i) 95 °C/10 min; (ii) 40 cycles of 95 °C/10 s, 60 °C/55 s. IAC is the internal amplification control and proves that the PCR reaction itself took place. If IAC is negative the reaction has to be repeated (Hutzler *et al.*, 2010). The yeast strains *Saccharomyces cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70 were used as a negative control. Target yeast, primer/probe sequence (5'-3') and references can be viewed in Table 3.

PCR fingerprinting

Yeast DNA was isolated using InstaGene Matrix (Biorad, Munich, Germany). Concentration of the DNA was measured with NanoDrop-1000 (Thermo Scientific, Wilmington, DE, USA) and adjusted to 25 ng/µl.

For RAPD analysis, the primer sequences (5'-GCT CGT CGC T-3′) were used according to

Tornai-Lehoczki and Dlauchy (2000). PCR parameters were: (i) 93 °C/3 min; (ii) 35 cycles of 93 °C/1 min, 38 °C/1 min, 72 °C/2 min; and (iii) 72 °C/5 min (Tornai-Lehoczki *et al*., 2000).

For GTG_5 analysis, the primer sequences (5′-GTG GTG GTG GTG GTG-3′) were used according to Healy *et al*. (2005). PCR parameters were: (i) 95 °C/5 min; (ii) 30 cycles of 95 °C/30 s, 40 °C/1 min, 72 °C/8 min; and (iii) 72 °C/16 min (Healy *et al*., 2005).

PCR was carried out using a thermal cycler (MasterCycler, Eppendorf, Germany). Typical PCR was performed with 12.5 µl RedTaq Master Mix 2× (Genaxxon, Ulm, Germany), 5 µl PCR water, 5 µl primer 21 or GTG_5 primer (Biomers GmbH, Ulm. Germany) and 2.5 µl template DNA with a total reaction volume of 25 µl. Amplicons were analysed using a microchip electrophoresis system: Agilent DNA 7500 kit (Agilent 2100, Agilent Technologies, USA). Fingerprint analysis was used to investigate the genetic relationships between strains. A dendrogram was built using the Bionumerics program (Applied Maths, Austin, USA).

Wort analysis

The composition and attributes of the used wort can be viewed in Table 4. To ensure standardized conditions for all trials, wort was manufactured from one batch of non-hopped wort extract (Doehler GmbH, Darmstadt, Germany) and deionized water. The pH value was adjusted to 5.2 using 10 M NaOH as the extract had a very low pH of 3.3. It was sterilized for 45 min at 100 °C. Free α-amino nitrogen was quantified using the MEBAK II. 2.8.4.1 method. Sugar composition was determined using high-performance liquid chromatography (HPLC) MEBAK II. 3.2.2.1.2 method. Final attenuation was determined using the MEBAK II. 2.9.3 method. The pH value was measured using ProfiLine pH 3210 (Xylem Inc., New York, USA).

Table 4. Wort attributes after sterilizing process used for fermentation and resistance tests

Specific gravity	**12.06 °P**
pH value	5.20
Sugar composition	Fructose 1.89 g/l Glucose 9.19 g/l Sucrose 3.80 g/l Maltose 55.48 g/l Maltotriose 15.41 g/l
Free α-amino nitrogen (FAN)	19.94 mg/100 ml

Hop/ethanol resistance

The investigated pure yeast strains were taken from wort agar slopes using an inoculation loop and incubated in 100 ml flasks containing 60 ml of wort (Table 4). The flasks were placed on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) and incubated for 1 day at a temperature of 27 °C (Salvadó *et al*., 2011). A cell count was then performed using the Cellometer Auto X4 (Nexcelom Bioscience LLC, Lawrence, MA, USA).

Hop resistance

Three 200 ml flasks containing sterile wort were adjusted to iso-α-acid concentrations of 0, 50 and 90 ppm (same in IBU). To adjust the concentration, a stock solution was mixed with 1 g of 30% iso-α-extract (Barth-Haas Group, Nürnberg, Germany), which was placed in a 50 ml flask. The flask was filled with ethanol (96% v/v) to 50 ml to dissolve the iso-extract. An aliquot of the stock was added to each wort to adjust the iso-α-acid concentration as needed.

Ethanol resistance

Two 200 ml flasks containing sterile wort were adjusted to ethanol concentrations of 5% (v/v) and 10% (v/v) by adding an aliquot of 96% (v/v) ethanol to the flasks.

Cross-resistance

Three 200 ml flasks containing sterile wort were adjusted to 0, 50 and 90 ppm iso-α-acid as explained above. An aliquot of ethanol was added to each flask to obtain an ethanol concentration of 5% (v/v) for each iso-α-acid concentration.

For each sample an Eppendorf tube was set up with 1 ml of the corresponding wort (ethanol or iso-α-acid or both added). The pure grown yeast strains were added to a total cell count of 100 000 cells/ml. Triplicates of 200 µl were taken from the Eppendorf tubes and transferred to a 96-well microtitre plate. Blank wort samples of 200 µl were also transferred in triplicate to reduce

the turbidity of the wort. The plate was sealed with a permeable plastic cover and placed inside the photometer. The temperature was set to 27 °C (Salvadó *et al.*, 2011). The optical density of the wells was measured at 600 nm every 10 min, followed by 8 min of heavy orbital shaking. The blank value density was subtracted from the measured density.

Phenolic off-flavour test

Coumaric, ferulic and cinnamic acid YM media

For the stock solution of ferulic and cinnamic acid, 1 g of the instant was diluted in 20 mL of 96 % (v/v) ethanol. For the stock solution of coumaric acid, 100 mg was dissolved in 10 mL of 96 % (v/v) ethanol. An aliquot of the stock solutions was added to the YM media containing agar at 45–50 °C under sterile conditions. The investigated pure yeast strains were taken from wort agar slopes and spread on the YM agar plate containing one of the described acids. TUM 68 was also taken into account to have a positive control.

Propagation

Investigated pure yeast strains were taken from wort agar slopes using an inoculation loop and inoculated into a 100 ml flask containing 60 ml of sterile wort. The flask was placed on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) and incubated for 2 days at an orbital agitation of 90 rpm and 27 °C. As described by Salvadó *et al.* (2011), 27 °C is the optimum growth temperature of *T. delbrueckii*. After 2 days the yeast-containing wort was transferred to a flask containing 500 ml of sterile wort. The flask was placed on the orbital shaker once again and incubated for 3 days at 27 °C at an orbital agitation of 90 rpm. Viability was measured using a Cellometer Auto X4 (Nexcelom Bioscience LLC) with propidium iodide solution to stain dead cells.

Fermentation

Fermentation tests were carried out using 2-litre sterile Duran glass bottles (Schott AG, Mainz, Germany) with a glass fermentation block, so that CO_2 could be controlled under sterile conditions. All fermentations were performed at triple determination with 2 litres of wort for each fermentation. Respective fermentation temperature was 27 °C. Fermentation was performed until no change in extract could be measured for two consecutive days. The pitching rate was 15×10^6 viable CFU/ml at a viability of at least 96% (v/v). The viability of the investigated yeasts was measured using a Cellometer Auto X4 (Nexcelom Bioscience LLC) with propidium iodide solution to stain dead cells.

Analysis of the produced beers

30 ml samples of each fermentation were withdrawn every day. The cell count was performed using the Cellometer Auto X4 (Nexcelom Bioscience LLC). The yeast was separated by a pleated filter and specific gravity was measured using DMA 35 N (Anton-Paar GmbH, Graz, Austria). The pH value was determined using the ProfiLine pH 3210 pH meter (Xylem Inc., New York, USA).

Final samples of 1 litre were withdrawn after 7 days and the following analyses were performed. Alcohol content was measured by an Alcolyzer Plus with DMA 5000 X sample 122 (Anton-Paar GmbH, Ostfildern, Germany). Fatty esters were determined by gas chromatography (GC) according to the protocol in Table 5. Fermentation by-products were determined using GC headspace (Table 6). TurboMatrix 40 Headspace parameters are displayed in Table 7. The amino acid content was quantified using the HPLC MEBAK II 2.8.4.1 method. Sugar composition was determined using the HPLC MEBAK II 3.2.2.1.2 method.

Table 5. Temperature protocol and column for GC fatty ester determination used in this study

Column	50 m 0.32 mm Phenomenex FFAP, 0.25 µm
Temperature protocol	1 min, 60 °C; 3 min, 220 °C (5 °C/min); 8 min, 240 °C (20 °C /min)
Detector temperature	250 °C
Injector temperature	200 °C

Table 6. Temperatures and column used for GC determination of fermentation by-products

Column	INNOWAX cross-linked polyethylene glycol, 60 m × 0.32 mm 0.5 µm
Oven temperature	200 °C
Detector temperature	250 °C
Injector temperature	150 °C
Injection time	4 s
Analysing time	17 min

Table 7. Temperatures and parameters of headspace sampling

Sample temperature	60 °C
Transfer temperature	130 °C
Needle temperature	120 °C
GC cycle	22 min
Thermosetting time	46 min
Pressurization time	1 min
Injection time	0.03 min

Sensory evaluation

All beer samples were tasted and judged by a sensory panel of 10 panellists with long-standing experience in the sensory analysis of beer and certified by the DLG (Deutsche Landwirtschafts-Gesellschaft e.V.). From initial trials with *T. delbrueckii* three main categories – fruity, floral and wort-like – were chosen as the main flavour categories produced by the investigated strains. Every category was judged from 0, meaning not noticeable, to 10: extremely noticeable. Secondly, a descriptive sensory evaluation was conducted, leaving it to the very experienced panellists to describe the flavour. Samples were given in triplicate in dark glasses with a three-digit code.

Results and discussion

The key parameters of beer production for yeasts are the capability to digest wort sugars and being able to grow in hopped wort (Hazelwood *et al.*, 2010; Bamforth, 2003). One more conceivable parameter is the capability of fermentation to a certain alcohol content. In terms of sugar utilization, the API 32-C test shows a wide variety in the 10 different yeast strains investigated (Table 8). This wide variety was mentioned by Kurtzmann *et al.* (2011). Looking at the sugars that are important for brewing (glucose, fructose, saccharose, maltose, maltotriose) (Narziß *et al.*, 1999, 2012) we can see that all strains are capable of fermenting glucose. Sugar analysis of the finished beer showed that all strains were also capable of fermenting fructose (Table 13). Furthermore, all of the yeast strains were able to ferment sucrose as seen in Table 9. This signifies that all of the investigated yeast strains have the enzyme invertase, which is required to convert sucrose into glucose and fructose (Alves-Araújo *et al.*, 2007). Looking at the main sugar of wort – maltose – one strain was found which was capable of utilizing it. The ability to ferment maltose indicates the presence of both a maltose transporter and the enzyme maltase (Goldenthal *et al.*, 1987). Looking at the sugar composition of the final beer, maltotriose was also utilized by T9. T9 appears to utilize many more sugars than the other strains. To ensure the purity of this strain, real-time PCR reactions with *Saccharomyces* species target sequences according to Hutzler *et al.* (2015) were performed, proving that no *Saccharomyces* cross-contamination had occurred in the T9 population (data not shown) (Hutzler *et al.*, 2015). Furthermore, single colonies were picked from YM agar and all colonies were identified as *T. delbrueckii* using the specific real-time PCR system shown in Table 3 (data not shown). All investigated strains besides T9 cannot ferment maltose or maltotriose, as proven by the sugar tests as well as the analysis of the sugar composition of the final beers.

The second main criterion was the capability of growing and fermenting in the presence of hops. All the strains were able to grow in IBU (international bitterness units) of 50 and 90 (Table 9). There are certain values of IBU that vary according to beer style. Wheat beer has 15–20 IBU, which results in 15–20 mg iso-α-acids/l, Pils has 30–38 IBU, and some highly hopped IPAs have up to 100 IBU (Bamforth, 2003). The results showed that the presence of iso-α-acids did have an influence on the growth of the investigated yeast strains. The presence of 90 IBU results in a longer log phase as well as a lower slope of log phase compared with 50 and 0 IBU. Fig. 1 shows the growth of T17 as an example of the influence of different IBU values (due to all strains exhibiting the same behaviour, the rest of the data is not shown). Significantly slower

Table 8. List of API 32C test results for the 10 investigated yeasts strains. Substrates that had a negative result for all strains are not shown

Yeast strain	T6	T9	T10	T11	T13	T15	T17	T18	T19	T20
GAL	–	–	+	–	+	+	–	+	–	–
SUC	+	+	+	+	+	+	+	+	+	+
LAT	+	+	–	+	–	+	+	+	+	+
RAF	–	+	–	+	–	+	+	+	+	+
MAL	–	+	–	–	–	–	–	–	–	–
TRE	–	+	–	+	–	–	+–	–		–
2KG	+	+	+	+	+	+	+	+	+	+
MDG	–	+	–	–	–	–	–	–	–	–
MAN	+	+	+	+	+	–	+	+	+	+
LAC	–	–	–	–	+	+–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–
SOR	+	+	+	+	+	+	+	+	+	+
GLY	+	+	+	+	+	+	+	+	+	+
PLE	–	+	–	–	–	–	–	–	–	–
MLZ	–	+	–	–	–	–	–	–	–	–
GNT	–	+	–	–	–	–	–	–	–	+
GLU	+	+	+	+	+	+	+	+	+	+
SBE	–	+	+–	–	+	–	–	+	+	+

Results of API ID 32c yeast identification: (+) positive, (−) negative, (+−) possible; LAT, lactic acid; RAF, D-Raffinose; MAL, D-maltose; TRE, D-trehalose; 2KG, 2-ketogluconate; MDG, methyl-αD-glucopyranoside; MAN, D-mannitol; LAC, D-lactose; 0, no substrate; SOR, D-sorbitol; GLY, glycerol; PLE, palatinose; MLZ, D-melezitose; GNT, potassium gluconate; GLU, D-glucose; SBE, L-sorbose; SUC, sucrose.

Table 9. Growth of the investigated yeast strains in wort with two different concentrations of iso-α-acids

IBU	T6	T9	T10	T11	T13	T15	T16	T17	T18	T19	T20
50	+	+	+	+	+	+	+	+	+	+	+
90	+	+	+	+	+	+	+	+	+	+	+

Growth (+) positive; (−) negative.

growth was reported at higher concentrations of iso-α-acids, as can be seen from Fig. 1. All the investigated yeast strains were able to grow in hopped wort and are thus able to ferment highly hopped worts from wheat beer to IPA.

To investigate the ability of the yeast strains to post-ferment a green beer, their growth and fermentation capacity was tested in wort containing 5 % (v/v) ethanol and 10 % (v/v) ethanol. None of the strains showed growth in 10 % (v/v) ethanol. 5 % (v/v) ethanol was only lethal for T6, as seen from Table 10. It is possible to post-ferment a green beer containing 5 % (v/v) ethanol for flavour purposes with all of these strains besides T6. Looking at the cross-resistance of ethanol and iso-α-acids of the investigated yeasts shown in Table 11, the strains T9, T11, T15, T17, T18 and T19 would be capable of post-fermenting highly hopped beers with an ethanol concentration of 5 % (v/v).

None of the investigated yeast strains showed any positive POF behaviour. However, the yeast strains did have different aromas, which could not be measured in the plates. It was described as a fruity yeasty flavour. These results show that none of the investigated yeast strains have the active enzyme to perform a decarboxylation of coumaric acid, cinnamic acid or ferulic acid (Coghe *et al.*, 2004; Scholtes *et al.*, 2014; Shinohara *et al.*, 2000). Therefore no off-flavour of this kind can be expected in the final beer (data not shown).

Propagation

At propagation, cell counts averaged from 140×10^6 cells/ml to 170×10^6 cells/ml, as illustrated in Fig. 2. T6 showed lower cell counts of 109×10^6 cells/ml. Viability measurements showed good conditions of

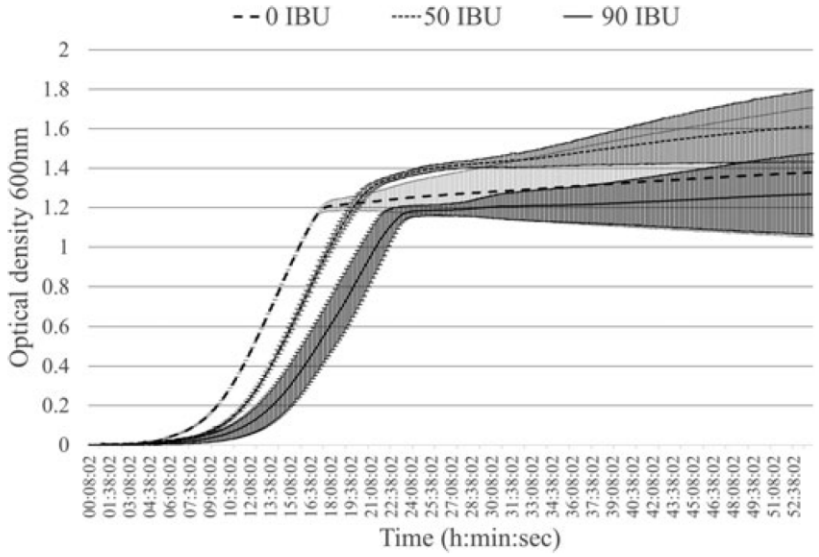

Figure 1. Growth of the investigated yeast strain T17 at different IBU values measured by the optical density at 600 nm in triplicate. Graphs show the mean of the triple measurements with standard deviation ($n = 3$)

Table 10. Growth of the investigated yeast strains in wort with two different concentrations of ethanol

Ethanol % (v/v)	T6	T9	T10	T11	T13	T15	T17	T18	T19	T20
5	–	+	+	+	+	+	+	+	+	+
10	–	–	–	–	–	–	–	–	–	–

Growth (+) positive; (–) negative.

Table 11. Growth of the investigated yeast strains in wort with two different concentrations of iso-α-acids and 5% (v/v) ethanol

IBU/ethanol % (v/v)	T6	T9	T10	T11	T13	T15	T17	T18	T19	T20
50/ 5	–	+	–	+	–	+	+	+	+	–
90/ 5	–	–	–	+	–	+	+	+	+	–

Growth (+) positive; (–) negative.

all strains from 98.8 % to 96.3 %. Only T11 had slightly lower viability at 95.3 %.

Fermentation trials

The fermentation trials were fermented until no change in extract was visible for 2 days. The percentage of fermented sugars (Table 12), as well as the final composition of the amino acids, was examined (Fig. 3). Utilization of different sugars by *Torulsporda delbrueckii* can vary from strain to strain, as described by Kurtzman *et al.* (2011). Fructose and glucose were fermented to over 90% by all the investigated yeast strains. Sucrose was fermented to about 70–80%. T9 fermented 94.8% of maltose and 58.9% of the total maltotriose. All other strains did not ferment maltose or maltotriose as predicted before.

In terms of amino acid anabolism and catabolism, all the investigated strains showed similar behaviour (only the data of T9 and T18 are shown in Figs. 3 and 4). Both pathways are very important for the content of aroma-active substances, such as higher alcohols, in the final beer (Vanderhaegen *et al.*,

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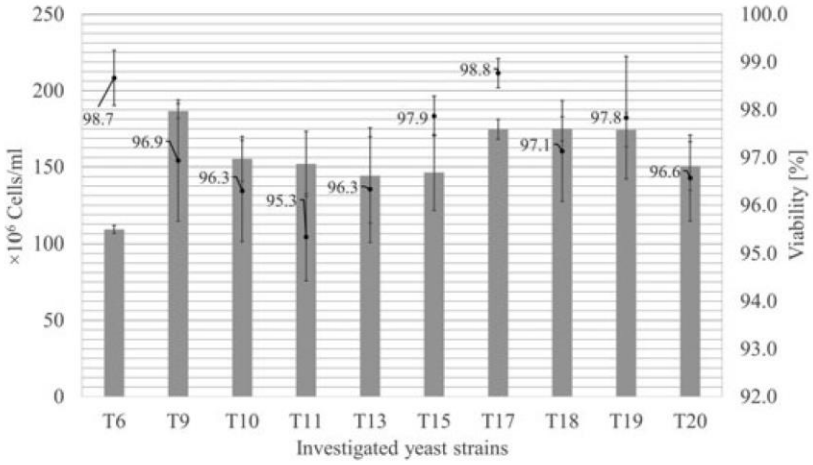

Figure 2. Results of propagation of all investigated yeast strains with cell count (10^6 cells/ml) on the left *y*-axis displayed by the grey bars, and viability (%) on the right *y*-axis displayed by black dots. The graph shows the means of triple measurements with standard deviation

Table 12. Mean percentage of wort sugar utilization during fermentation from wort to finished beers measured in triplicate; confidence level 95%

	T6	T9	T10	T11	T13	T15	T17	T18	T19	T20
Fructose (%)	93.2 ± 0.2	92.3 ± 0.6	91.54 ± 0.7	88.0 ± 0.0	91.6 ± 0.2	90.2 ± 0.2	84.5 ± 0.1	96.4 ± 0.0	93.6 ± 0.0	88.1 ± 0.7
Glucose (%)	96.6 ± 1.1	96.2 ± 0.1	97.0 ± 1.5	96.6 ± 0.1	97.3 ± 0.2	95.5 ± 0.4	94.5 ± 0.1	95.4 ± 2.0	94.7 ± 0.6	89.6 ± 3.5
Sucrose (%)	82.4 ± 0.7	86.4 ± 6.4	79.0 ± 0.9	95.0 ± 1.4	84.6 ± 0.1	75.2 ± 0.2	78.3 ± 1.5	72.0 ± 0.8	73.7 ± 0.2	84.7 ± 0.4
Maltose (%)	3.3 ± 0.2	94.8 ± 1.6	5.8 ± 2.0	6.0 ± 1.8	1.8 ± 0.7	0.3 ± 2.0	0.9 ± 0.9	02.5 ± 4.1	0.7 ± 0.6	0.3 ± 1.9
Maltotriose (%)	3.0 ± 1.2	58.9 ± 2.8	1.6 ± 1.3	4.2 ± 2.7	0.5 ± 0.2	1.3 ± 0.6	2.4 ± 1.9	0.1 ± 0.4	0.1 ± 1.0	3.6 ± 1.0

2003; Procopio *et al.*, 2011). Higher alcohols such as propanol, isobutanol, isoamyl alcohol, phenyl ethanol and 2-methyl butanol are formed from amino acids via the catabolic pathway (Ehrlich pathway). The amino acids are transaminated and decarboxylated to α-keto acids, which are then reduced to higher alcohols (Verstrepen *et al.*, 2003a; Hazelwood *et al.*, 2008). α-Keto acids can also be formed in a *de novo* synthesis from carbohydrates via pyruvate and then decarboxylated to aldehydes, which can be reduced to higher alcohols (Hazelwood *et al.*, 2008).

Fig. 3 shows the distribution of amino acids in the fermentation of T9 before and after fermenting the wort. The initial values of amino acids vary between the yeast strains. This is due to the fact that the cell count of the different strains varied. This resulted in the starting parameters being slightly diluted as a result of more wort being added, which contained yeast cells. The beers fermented with T9 had the lowest end-concentration of amino acids. This was expected owing to its longer fermentation time and higher sugar uptake. It also formed larger quantities of higher alcohols than the other strains, as seen in Table 15. Fig. 3 also shows that T9 formed arginine, as previously reported for many *Saccharomyces* and non-*Saccharomyces* strains by Romagnoli *et al.* (2014). T18 shows very little uptake of amino acids apart from alanine. Tyrosine was formed, which is shown at a significantly higher value after fermentation in Fig. 4. Summarizing the final levels of amino acids, we can say that every green beer had comparable high levels of every amino acid. Post-fermentation of the low fermented beers by a *Saccharomyces* yeast would thus be possible as many amino acids are left to propagate and to ferment until final attenuation.

All investigated yeasts produced alcohol, as seen in Table 13. The alcohol content of all the beers fermented by the maltose-negative strains was close to 0.94 % (v/v). T9 fermented until

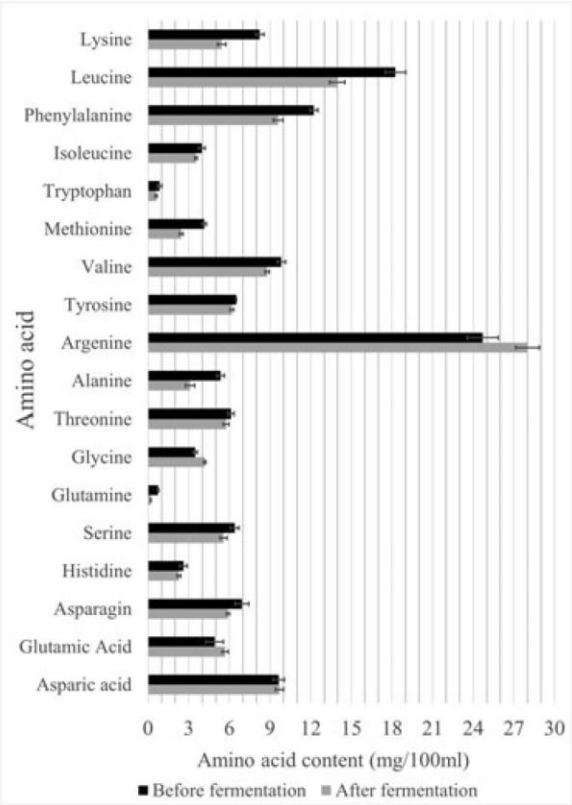

Figure 3. Amino acid content (mg/100 ml) of pitched wort and beer fermented by T9 measured in triplicate at the start and end of fermentation, with standard deviation

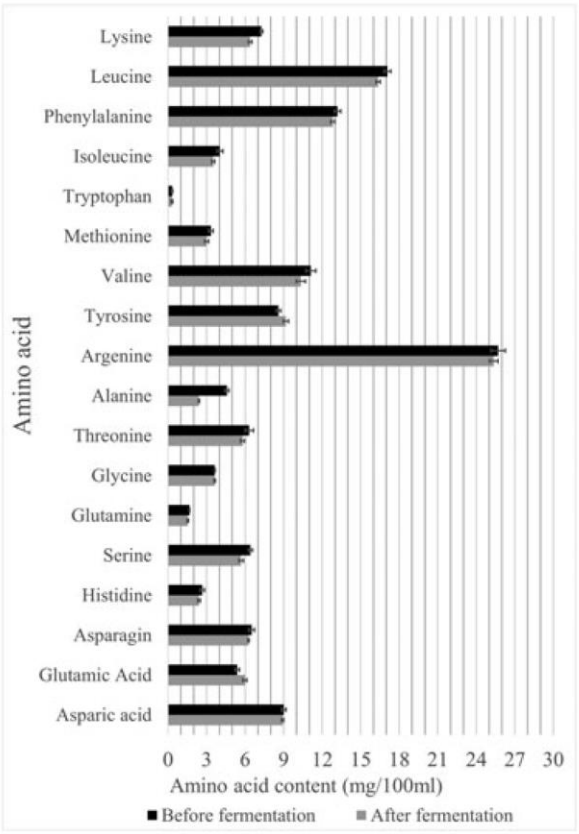

Figure 4. Amino acid content (mg/100 ml) of pitched wort and beer fermented by T18 measured in triplicate at the start and end of fermentation, with standard deviation

producing a final alcohol content of 4.0 % (v/v), which is very close to the alcohol content of an average beer with an extract of 12 °P and a residual extract of approx. 4 °P.

Due to the fermentation of the maltose-negative yeast strains ending quickly, very low concentrations of yeast cells in suspension of supernatant were detected after 24 h fermentation (data not shown). However, T9 increased to 70 million cells/ml on day 2, dropping about 10 million cells/ml each consecutive day of fermentation. A good clearance of green beer after fermentation was visible in all fermentation vessels. With the exception of T15, all investigated yeasts were visible as a fine dust at the bottom of the fermentation vessels. T15, however, formed cell fusions in the shape of pellets with an external diameter of about 0.2 cm. T15 can be described as a fast-flocculating yeast, whereas all other investigated yeasts can be described as low-flocculating yeasts. The pH value of all the young beers dropped in the first 24 h to below pH 4.2, which is necessary because the low pH, the produced ethanol, the hop-derived antimicrobial compounds and the carbon dioxide are hostile to the growth of many different bacteria, especially Gram negative, which could spoil the beer (Bokulich *et al.*, 2012).

Extract of the wort fermented by T9 decreased constantly every day by 1 °P, ending on day 7 at 4.5 °P. All other strains reached an average of 10 °P at 48 h fermentation. As a result of only fermenting glucose, fructose and sucrose, this is the predicted result of their fermentation.

Volatile compounds contents of the different beers fermented with *T. delbrueckii* showed strong distinctions among the different groups of higher alcohols, esters and acetate esters (Table 14). Higher alcohols are mostly synthesized using the Ehrlich pathway in the presence of sugars and amino acids (Hazelwood *et al.*, 2008). 2-

Table 13. Mean and standard deviation of the alcohol content of green beers fermented by the investigated yeasts ($n = 3$)

Yeast	T6	T9	T10	T11	T13	T15	T17	T18	T19	T20
Ethanol % (v/v)	0.87 ± 0.01	4.00 ± 0.01	0.83 ± 0.01	0.87 ± 0.02	0.89 ± 0.01	0.94 ± 0.01	0.89 ± 0.01	0.87 ± 0.02	0.9 ± 0.02	0.91 ± 0.01

Phenylethanol, *n*-propanol, *i*-butanol and amyl alcohols were the main objectives of the measurement. The content of 2-phenylethanol produced by T9 was almost twice as high, at 23.7 mg/l, as that of all the other beers studied. T6, T10, T13 and T15 are still above the odour threshold of 10 mg/l, which contributes to a sweet, rose and floral aroma (Guth, 1997; Etschmann *et al.*, 2015). The *n*-propanol and *i*-butanol contents were very high in the samples fermented by T9. All other strains showed amounts below the average content of top-fermented and also of bottom-fermented beers, as shown in Table 15. The amyl alcohol content of 64.8 mg/l in the beer fermented by T9 was three times higher than that of all other investigated yeast strains. The odour threshold for amyl alcohol, which is considered to be a solvent like brandy aroma, was 50–70 mg/l (Pires *et al.*, 2014). All the investigated yeasts except T19 produced almost half of the odour threshold of amyl alcohols.

Apart from higher alcohols, two further major volatile compounds that contribute to the aroma of beer are esters and acetate esters (Verstrepen *et al.*, 2003b; Lettisha *et al.*, 2013; Renger *et al.*, 1992). Esters are synthesized in a reaction between alcohol and medium-chain fatty acids (Plata *et al.*, 2003). Acetate esters are formed from acetyl-CoA and a higher alcohol provided by the enzyme alcohol acetyltransferase (Verstrepen *et al.*, 2003a). The total ethyl acetate concentration of the beer fermented by T9 was 23.4 mg/l – four times higher than the concentration of ethyl acetate in any other beer fermented by the investigated yeast strains. In 2002, Zohre and Erten (2002) reported that concentrations of ethyl acetate below 50 mg/l do not contribute to flavour. However, the synergy of different volatile compounds could contribute to the total flavour, as suggested by Sterckx *et al.* (2011). The total amount of isoamyl acetate was below the detection level of 0.1 mg/l in all beers. Investigations by Hernández-Orte *et al.* (2008) showed that the production of isoamyl acetate by *T. delbrueckii* is extremely slight. The concentration of ethyl caproate, ethyl caprylate and ethyl caprate, which is known for a green apple-like flavour, did not reach higher than 0.01 mg/l in either of the beers (data not shown). Diacetyl values of all beers were above the threshold of 0.1 mg/l. Some beer styles lack this aroma compound, which is known for its buttery flavour. Direct analysis of the beers meant that there was no maturation and therefore no diacetyl reduction during maturation. Two common German beer styles (top fermented and bottom fermented) analysed by Narziss and Back (2005), Dittrich (1993) and Renger *et al.* (1992) were added to the data to provide a comparison.

To evaluate the flavour of the beers fermented by different strains, a panel of 10 trained and experienced beer tasters judged the beers first by three descriptors: fruity, wort-like and floral. Each descriptor was awarded a value from a scale of 0 (very low threshold) to 10 (very high threshold). No significant difference ($\alpha = 0.05$) was found by the sensorial testing panel among the fruity, floral and wort-like attributes in any of the triplicates. This signifies that the triplicates were very equal in these attributes. Analysis of variance (ANOVA) showed a high significant difference ($p < 0.05$) between the beers produced by the different yeast strains (O'Mahony, 1986). As shown in Fig. 5, beers produced by T9 and T17 were judged to be the most fruity flavoured beers out of all the beers fermented by the investigated yeast strains. Owing to the high amount of maltose and maltotriose in all the beers except for those fermented by T9, there was always a slight wort-like flavour. This is also because the wort was not hopped, to obtain the pure flavour of the yeast. T20 and T18 produced low flavour thresholds, followed by T19, which means that the beers had a rather neutral smell and taste.

Descriptive sensory evaluation by the panellists showed an overall tendency to honey and pear-like flavours. Beers fermented by T11 and T19 were also judged to be plum-like. T9, T17 and T13 were described as having strong citrus fruit flavours.

The GTG 5 and RAPD 21 fingerprint systems showed very similar results for all strains except for T19 (Fig. 6). Differentiation by the GTG 5

Table 14. Fermentation by-products measured in the produced beer of the different investigated yeast strains stated by mean and standard deviation ($n = 3$). Two average German beer styles were added for comparability

		Average German beer[a]											
Fermentation by product		**Top fermented**	**Bottom fermented**	**T6**	**T9**	**T10**	**T11**	**T13**	**T15**	**T17**	**T18**	**T19**	**T20**
Ethyl acetate(mg/l)	m	29	19	3.83	23.40	2.23	4.56	5.33	2.76	5.96	3.76	3.90	4.36
	s			±0.15	±0.43	±0.15	±0.20	±0.20	±0.05	±0.11	±0.23	±0.17	±0.15
Amyl alcohols(mg/l)	m	50-70	38–100	28.03	64.83	25.26	25.93	29.10	24.10	28.36	20.73	15.00	18.60
	s			±1.33	±0.55	±1.10	±0.41	±1.03	±0.40	±1.12	±2.08	±0.36	±0.10
Diacetyl(mg/l)	m	<0.1	<0.1	0.31	0.43	0.130	0.14	0.10	0.15	0.12	0.15	0.20	0.14
	s			±0.02	±0.09	±0.01	±0.00	±0.01	±0.01	±0.01	±0.00	±0.03	±0.01
Decanoic acid(mg/l)	m	1.9	0.2–1.8	2.00	0.02	0.67	0.49	1.13	0.08	0.67	2.06	1.07	0.35
	s			±0.60	±0.01	±0.05	±0.02	±0.24	±0.01	±0.06	±0.92	±0.36	±0.07
2-Phenylethanol (mg/l)	m	31	8–25	12.83	23.70	15.83	8.13	13.90	11.80	8.93	5.40	5.03	5.26
	s			±0.32	±1.13	±0.90	±0.25	±0.96	±0.20	±0.20	±0.45	±0.23	±0.15
n-Propanol(mg/l)	m	21	7–16	4.83	17.06	3.03	4.90	4.13	5.70	3.66	2.93	2.73	2.33
	s			±0.40	±0.40	±0.23	±0.45	±0.32	±0.10	±0.20	±0.60	±0.05	±0.05
i-Butanol(mg/l)	m	28	5–20	3.46	19.96	2.36	4.46	3.56	2.10	3.30	1.36	2.80	4.00
	s			±0.15	±0.20	±0.28	±0.05	±0.15	±0.00	±0.10	±0.25	±0.20	±0.10
Hexanoic acid(mg/l)	m	1.5	1–5	0.20	0.07	0.16	0.18	0.20	0.13	0.13	0.23	0.13	0.13
	s			±0.02	±0.01	±0.00	±0.00	±0.02	±0.01	±0.00	±0.02	±0.00	±0.00
Octanoic acid(mg/l)	m	3.2	5–10	0.59	0.11	0.36	0.49	0.57	0.33	0.42	0.74	0.39	0.30
	s			±0.08	±0.02	±0.02	±0.02	±0.05	±0.03	±0.01	±0.09	±0.06	±0.03

m, mean; s, standard deviation.
[a]Dittrich (1993); Renger *et al.* (1992); Narziss *et al.* (2005).

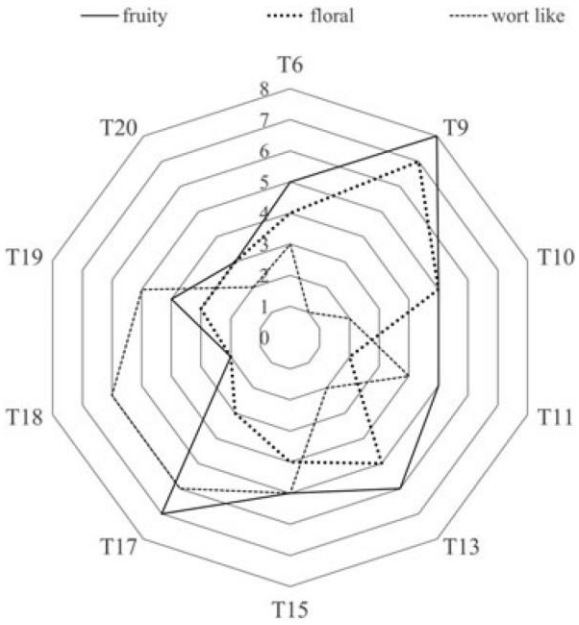

Figure 5. Distribution of the three different attributes – fruity, floral and wort-like – of beers produced by the 10 different yeast strains

fingerprint is more discriminative than the RAPD 21 method. Single strains can be differentiated (e.g. T15 and T6 from the clade T11, T13, T9 and T10). The T9 strain, which differs physiologically (sugar metabolism), shows similar fingerprint patterns to T10, T11 and T13. No physiological properties are reflected by fingerprint clustering. For quality-control purposes, research into other fingerprint methods with higher discriminative power can be considered.

Outlook

The results presented in this study show that *Saccharomyces* is not the only genus that can be used for brewing. Many traditional beverages from countries all over the world are partly made by yeasts that do not belong to the *Saccharomyces* genus. So-called mixed fermentations can produce various results with different aromas (Vanderhaegen *et al.*, 2003). Screening these yeasts for their ability to brew beer and finding new yeasts that are capable of doing so is the main goal of our future research. In terms of the screening system, some adjustments need to be made regarding propagation and sugar utilization tests. In addition to screening, a large variety of tests covered many of the essential beer attributes and requirements. All the investigated yeast

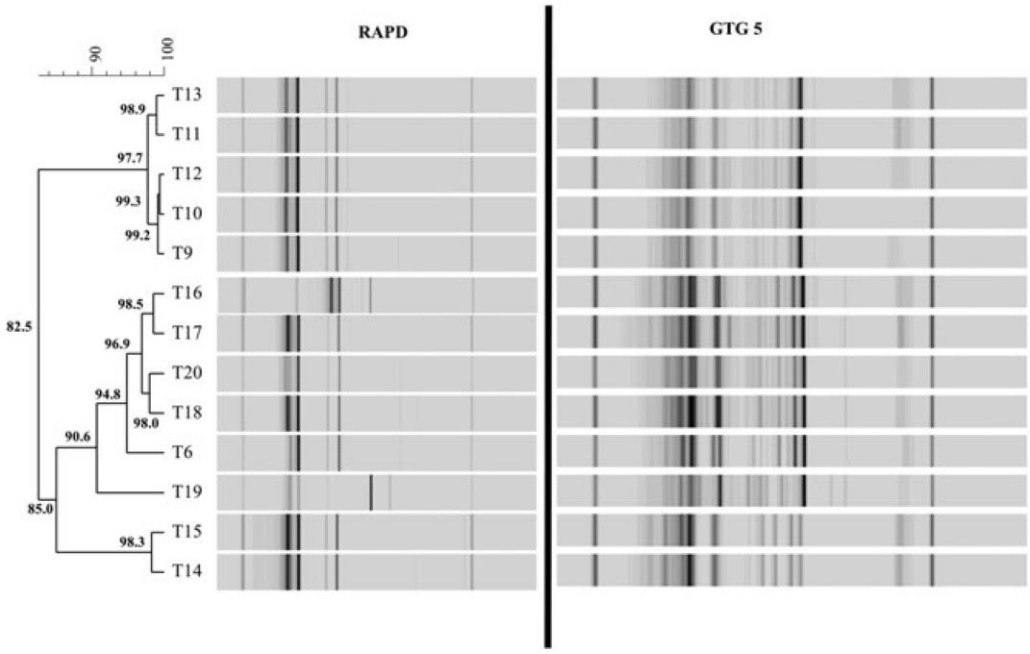

Figure 6. Neighbour-joining tree of the *Torulaspora* species resulting from cluster analysis of GTG 5 and RAPD 21-PCR patterns

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strains were able to ferment the used wort to a certain extent. T9 was discovered to be a yeast strain that could produce a beer with a rich fruity and floral flavour. It fermented all the necessary wort sugars in 7 days, forming 4% (v/v) ethanol and a large variety of flavour-active compounds. T17 and T13 fermented only glucose, fructose and sucrose but produced a rich fruity flavour. They can therefore be used to produce low-alcohol beers. Adjusting the wort to a lower sugar content or changing the wort in terms of its sugar composition could lead to fruity, low-alcoholic beers, as suggested by Meier-Dörnber *et al.* (2015). As all *T. delbrueckii* strains investigated in this study used very little of the available amino acids and were able to grow in hopped wort, post-fermentation with a *Saccharomyces* strain could be possible and will be investigated in further research. This should produce higher levels of volatile flavour compounds, as shown by research into post- and mixed fermentation of wine, and it has been proposed by Vanderhaegen *et al.* that this may also be true for beer (Vanderhaegen *et al.*, 2003; Zott *et al.*, 2008; Fleet, 2008).

Optimum temperatures of future new yeasts for growth as well as fermentation optimizations will be investigated. Larger trial fermentations in cylindroconical fermentation vessels need to be conducted to prove the brew ability in larger batches because of the associated higher pressure. The ultimate aim is to find a new yeast that breweries can use to produce a fruitier kind of beer. Matching hop types for *T. delbrueckii* beers might be an aim for new research.

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Part 3	2.4 Optimization of beer fermentation with a novel brewing strain *Torulaspora delbrueckii* using response surface methodology

A previous publication "Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as a model" resulted in the discovery of a strain T9, which seemed to have potential as a novel brewing strain. To improve the fermentation performance as well as flavor forming of this particular strain, a response surface methodology was applied. Varied parameters were fermentation temperature (15-25 °C), and pitching rate (50×10^6 - 120×10^6 cells/mL). Fermentations were carried out in 2 L glass bottles using diluted wort extract (from 62 °P to 12.5 P) from one large batch to ferment at standardized conditions. The fermentation onset, total ester content, total higher alcohol content, as well as flavor assessments as honey-like, blackcurrant-like and wine-like were defined as responses. Before fermentation, the timeframe of propagation was investigated to be able to pitch the yeast at its highest vitality, viability and cell concentration. Therefore, three 15 L glass propagators with a stirring system and sampling pumps were incubated with 5×10^6 cells/mL of T9 and cell count, vitality and viability was determined every 4 hours. In addition, a wort-oxygenation growth test was performed to investigate the optimal level of oxygenation of the wort prior to pitching (0.2, 5, 10, 15 and 20 mg/L dissolved oxygen).

The optimal time to pitch the yeast was found to be after 28 hours of propagation at a total cell count of 400×10^6 cells/mL and high vitality. A wort aeration test showed 10 mg/L dissolved oxygen to be sufficient. Response surface methodology showed significant strong changes in the flavor profile at varying temperatures but low changes at different pitching rates. The flavor was found to change from strong honey-like at low temperatures (15 °C) to blackcurrant-like at temperatures of about 20 °C, to wine-like at 25 °C. When evaluating the responses, a combination of 60×10^6 cells/mL pitching rate and 20 °C fermentation temperature was predicted to be the optimal combination. In addition, three 50 L fermenters were incubated at a pitching rate of 60×10^6 cells/mL and at 20 °C and values of esters, higher alcohols and flavor assessment were compared with the predicted values. Predicted and measured values were found to be almost equal.

Authors/Authorship contribution:

Michel M.: Literature search, writing, data creation, study conception and design; **Meier-Dörnberg T.**: critical review (fermentation), supported statistical analysis tasting; **Jacob F.**: Supervised the project; **Haselbeck K.**: Data analysis and interpretation; **Schneiderbanger H**.: concept of propagation; **Zarnkow M.**: Support in the statistical analysis of data (Design Expert); **Hutzler M.**: Supported the creation of research plan, critical content review

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PEER-REVIEWED PAPER

Optimization of Beer Fermentation with a Novel Brewing Strain *Torulaspora delbrueckii* Using Response Surface Methodology

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ABSTRACT

The use of novel brewing strains coming from the non-*Saccharomyces* sector adds new challenges but also new desirable aromas to beer. Some research teams have focused on screening new yeast species from biodiversity for their fermentation potential. The next step in creating novel brewing strains is the optimization of the fermentation process for the actual production of beer. Here a response surface methodology was used to adjust the fermentation process with a strain (T9, *Torulaspora delbrueckii*) found for potential use in a previous study. An oxygenation rate test was performed to investigate the oxygen requirement of the strain. Also, an evaluation of volatile flavor compounds and final flavor judgment by trained panelists were undertaken. The fermentation conditions were chosen according to prior testing at varying fermentation temperatures between 15 and 25°C and pitching rates due to relatively small cell sizes of between 50×10^6 and 120×10^6 cells/mL. Further, the optimal time of pitching from propagation in connection with viability, vitality, and cell count was investigated. The wort used was diluted to 12.5°P from wort extract for standardized conditions. Wort oxygenation until 10 mg/L of dissolved oxygen was found to be sufficient. Propagation cell counts reached up to 400×10^6 cells/mL in 28 h before viability decreased. Fermentation at 20°C and a pitching rate of 60×10^6 cells/mL led to the most desirable beer with a blackcurrant and honey-like flavor. A shift of flavor was found from honey-like at low temperatures to wine-like at higher temperatures.

Keywords: *Torulaspora delbrueckii*, Alternative yeast, Nonconventional yeast, Beer

The interest in using novel brewing yeast strains selected from the immense biodiversity of yeast for the production of beer has been growing in recent years (7,24,27,30,47,56). The *Saccharomyces* genus has been the main brewing yeast in the past decades and will surely remain the main yeast for large breweries with high production volumes (28). However, using an unconventional non-*Saccharomyces* yeast offers a unique selling point for craft brewers to distinguish themselves from others (7). Researchers from around the world have therefore started to think outside the box of *Saccharomyces* and have focused instead on finding non-*Saccharomyces* yeast with potential brewing ability (7,11,20,29,49). Many studies of screening and finding new brewing strains have been published (11,20,30). However, after the challenge of finding new strains it is necessary to actually optimize and control these strains for brewing. In contrast to *Saccharomyces* brewing yeast, wild yeasts have not undergone domestication for decades and therefore have many challenging properties that need to be considered (28,46).

The first challenge is to propagate viable and vital yeast for a predictable and consistent fermentation (8,13,18,22,50). The physiology of the pitching yeast is highly important owing to the close relation to cell growth and, consequently, fermentation performance and the production of desired and undesired flavor compounds (40,44). Viability describes the percentage of live cells (should be approximately 95–98%) within a population and is mostly measured by bright-field imaging, for example, using methylene blue or fluorescent staining (e.g., propidium iodide) (5,8,22,55). Vitality describes the metabolic activity of yeast and can be measured by various methods (e.g., acidification power test [18], formation and release of carbon dioxide [32], or intracellular pH value [54]). Because all of these methods measure different aspects, no general definition encompassing all the results can be given for vitality. Every method gives a different unit of measurement, which is defined in certain ranges to identify a yeast population that will contribute to a strong and predictable fermentation (18,32,54). The aim of propagation is further to provide high terminal cell counts (approximately $100–200 \times 10^6$ cells/mL) in the budding phase to allow maximum ratios for pitching and fast growth of the population once pitched (8). The propagation temperature should be kept close to fermentation temperature (variation approximately 1°C) to ensure the pitching yeast is not exposed to temperature stress (4,8). To minimize the risk of contamination, which is more likely in the propagation phase owing to optimum growth conditions, the propagation period should be kept as short as possible (4,8).

After propagating viable and vital pitching yeast, the initial fermentation parameters should be kept constant and at an optimum level to produce a high-quality product and to ensure

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a maximum conversion of wort ingredients and an efficient fermentative productivity at a predictable time (4,8,34). The main fermentation control parameters that influence the quality and consistency of the produced beer as well as the physiology of the pitching yeast and the composition of the used wort are as follows: 1) oxygenation of the wort, 2) pitching rate, and 3) fermentation temperature. For the most part these parameters vary by the type of beer and yeast used in the process (4,8,13,34). These parameters, which already vary for brewing yeast strains, are unknown for the fermentation of wort with *Torulaspora delbrueckii*. The impact and variation of brewing yeast strains will be explained in the following to provide an orientation to the values used in the further study.

1. The oxygenation of wort mostly impacts yeast growth (10,35). Directly related to the growth of a brewing yeast population of course is the rate of fermentation (3). The yeast cells need a certain amount of oxygen to produce sterols and unsaturated fatty acids that are needed to form new cell membranes (3,4). For bottom-fermenting yeast, a dissolved oxygen (DO) level of 8–9 mg/L of DO is recommended. Lower amounts of between 4.5 and 6 mg/L of DO are suggested for top-fermenting yeast. A concentration of below 4 mg/L of DO results in decreased fermentation speed and yeast growth (4,33).
2. The pitching rate again depends on the yeast used and the original gravity of the wort (5,16). If yeast is exposed to high original gravity wort (>13°P), the chosen pitching rate is normally higher than an average original gravity wort (<13°P) (48). Overall it can vary between 5×10^6 and 30×10^6 cells/mL, and the pitching rate of bottom-fermenting yeast is mostly chosen to be higher than that of top-fermenting yeast owing to lager yeasts' lower fermentation temperature (4,16,26,48). Higher pitching rates lead to higher fermentation rates (16), an increase of esters and fusel alcohols, and lower acetaldehyde concentrations (26). Especially for non-*Saccharomyces* yeast, the pitching rate may depend on the cell size. Size is related to the surface of the cell, which determines the amount of nutrition it can transport into the cell (4). Most non-*Saccharomyces* yeast strains have comparably smaller cells than the average brewing yeast (approximate mean cell diameters: *Saccharomyces cerevisiae*, 8 µm; *T. delbrueckii*, 3 µm [53]), which suggests that higher amounts of cells are needed (4,53). For the species *T. delbrueckii* there is little knowledge and experience on the pitching rate other than some minor reports (11,29,49).
3. The fermentation temperature is mostly chosen according to the brewing strain used (4,34,36,43). Varying the temperature makes it possible to change the yeast growth and therefore the fermentation speed. Too high temperatures, however, can harm the yeast or cause loss of volatile flavor compounds by temperature or gas stripping (9). Higher temperatures also result in higher amounts of higher alcohols and esters (51). If a top-fermenting yeast is used, the selected fermentation temperature is between 18 and 25°C (4,5,9,33). Fermentation with a bottom-fermenting yeast is mostly performed at temperatures between 6 and 16°C (9,33). For the species used in this study, *T. delbrueckii*, fermentation temperatures between 20 and 27°C have been reported (11,29,49).

The aim of this study was to combine the variation of pitching rate and fermentation temperature using a response surface method (RSM, central composite design). This method has been partially used to optimize malting (57), produce alcohol-free beer (39), and produce 4-vinylguaiacol and 4-vinylphenol (12). It has not yet been used to optimize the fermentation performance of a non-*Saccharomyces* yeast in brewing. RSM was applied to discover the optimum combination of the fermentation parameters explained earlier, which were then used in a scaled-up experiment by fermentation of 50 L in triple determination to find out if the fermentation could be predicted. Optimum oxygen demand was previously investigated following a modified method of Jakobsen and Thorne (25).

Materials and Methods

Yeast Strain and Wort

After screening 10 *T. delbrueckii* strains for their brewing ability by testing their sugar metabolism, phenolic off-flavor formation, hop and ethanol resistance, and fermentation of a standardized all-malt wort, one strain (T9, DSM 70504) was chosen for process optimization (DSM indicates Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). The strain had a good ability to ferment all the necessary wort. No major influence by either ethanol or hop acids could be observed. Good flavor formation and fermentation performance of the all-malt wort were also discovered (29).

For all the trials, the standardized wort used was diluted to 12.5°P from the wort concentrate of one large batch (Doehler GmbH, Darmstadt, Germany). Deionized water was used for the dilution. The original gravity was adjusted to 12.5°P for every trial. After preparation, the wort was sterilized at 100°C for 45 min.

Propagation

Three 15 L glass propagation devices were used to produce the required amount of viable and vital yeast for the fermentations. One propagation device included a glass tank filled with 10 L of wort sealed sterile, and a stirrer (Eurostar 60 digital, IKA, Staufen, Germany) with two stirring blades at ground and middle level set to 90 rpm. Aeration was performed with a sterile air filter (Minisart HY 0.2 µm, Sartorius, Göttingen, Germany), a D2 P1 NS29 filter disc (Glastechnische Werkstatt Dieter Verhees, Neuss, Germany), and a time-controlled pump (DULCOflex DF4a, ProMinent, Heidelberg, Germany) aerating the wort with 0.9 L of sterile air every 5 min (35) (Fig. 1). The entire propagation device was sterilized at 100°C for 45 min prior to use. The temperature was set to 20°C in respect to the fermentation temperatures at around 18–25°C depending on the RSM set values. Sterile inoculation was performed for each device with 5×10^6 cells/mL of *T. delbrueckii* (T9). The strain was inoculated under sterile conditions 48 h before at 24°C in a 1 L flask of sterile wort (500 mL) using a pure culture taken from slant agar (4). Cell count, pH value, and the extract and ethanol concentration were regularly monitored. Sampling was performed with a sampling pump (DULCOflex DF4a), as shown in Figure 1.

Measurement of Vitality and Viability

The vitality was measured according to a method of Müller-Auffermann et al. (32), modified owing to smaller cell size of *T. delbrueckii* and therefore the different fermentation rate dependent upon the cell count and surface. The original method measured the rise in pressure of a wort inoculated with 65×10^6 cells/mL to 1 bar. The modified method used 1.5 g of yeast as well as a pressure rise to 690 mbar. Prior to testing, 300 mL

of thin yeast slurry was centrifuged at 700 × *g* for 10 min at 20°C. The supernatant was discarded, and yeast was washed with 300 mL of 20°C deionized water by 30 s of vortexing with a Vortex-Genie 2 mixer at level 8 (Scientific Industries, New York, NY, U.S.A.). Three 100 mL Duran glass bottles containing 80 mL of sterile wort (12.5°P, pH 5.4) tempered to 25°C were inoculated with 1.5 g of the washed yeast and were placed on a magnetic stirring system at 300 rpm inside an incubator at 25°C. The bottles were sealed with a wireless automatic system for fermentative gas production monitoring (GMP, ANKOM Technology, Macedon, NY, U.S.A.) to detect the rise in pressure until it reached 690 mbar. The time taken to reach 690 mbar was measured to calculate the vitality. Viability was measured using a Cellometer Auto X4 automated cell counter (Nexcelom Bioscience, Lawrence, MA, U.S.A.) with propidium iodide solution (propidium iodide concentration, 2 g/L) for the fluorescence staining of dead cells.

Oxygen Demand

The method of Jakobsen and Thorne (25) was slightly modified to investigate the oxygen demand of the strain T9 (DSM 70504) (25). Therefore, 4.5 L of 12.5°P wort diluted with deionized water from standardized wort extract (Doehler GmbH) was divided up into five 1 L Duran glass bottles containing 0.9 L of wort. After preparation, the bottles were sterilized at 100°C for 45 min. The bottles were hermetically sealed directly after sterilization to keep the low level of oxygen. The wort was oxygenated using a sterile air filter (Minisart HY 0.2 µm, Sartorius) with pure oxygen (Linde AG, Munich, Germany), a D2 P1 NS29 filter disc (Glastechnische Werkstatt Dieter Verhees), and a flow meter (set to 50 mL/min at 1 bar). Aeration was performed until a DO content of 5, 10, 15, and 20 mg/L of DO was reached. One sample containing 0.2 mg/L of DO was used as the control. The samples were incubated with 60 × 10^6 cells/mL of strain T9, divided up into three 500 mL Duran glass bottles and sealed with a fermentation lock.

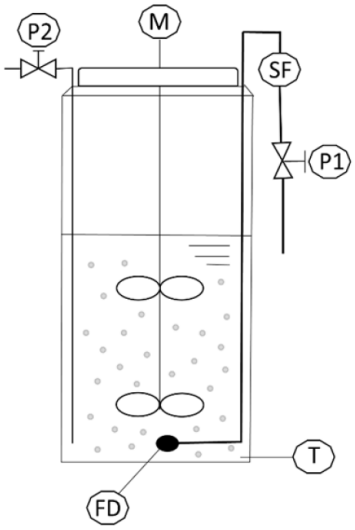

Figure 1. Propagation device for *Torulaspora delbrueckii* propagation. M = motor for agitator; P1 = time controlled aeration pump; P2 = sampling pump; T = temperature transmitter; FD = filter disc; and SF = sterile air filter.

They were then weighed to investigate the loss of CO_2 over 10 days. Loss of CO_2 indicated the fermentation of wort sugars. According to Balling, 2.0665 g of extract is converted into 1 g of alcohol, 0.11 g of yeast biomass, and 0.9565 g of CO_2 (6). The CO_2 loss, which occurs when fermenting with a usual air lock, can then be directly related to the degree of fermentation because the CO_2 can exit the bottle. Bottles were placed on a WiseShake orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany) at 80 rpm for 10 days at 26°C. The weight of the samples was determined every 24 h to calculate the attenuation by loss of CO_2.

Experimental Design and RSM

The pitching rate and fermentation temperature were adjusted as calculated by Design Expert 10 response surface software (Stat-Ease, Minneapolis, MN, U.S.A.). A central composite design was chosen with two numerical independent factors. Factor one, pitching rate range, was calculated for a high of 100 × 10^6 cells/mL and a low of 50 × 10^6 cells/mL. The cell count was performed with the Cellometer Auto X4 automated cell counter (Nexcelom Bioscience). Factor two, fermentation temperature range, was set to a high of 25°C and a low of 18°C. The pitching rate and temperature ranges were chosen according to prior results of a response surface investigation that used between 10 × 10^6 and 70 × 10^6 cells/mL and between 15 and 32°C (31). The software predicted a total of 17 experiments in one block, with nine replicates of the central point to investigate the responses (Table 1). Responses to the onset of fermentation were measured by the loss of total CO_2 after 48 h. Solubility of CO_2 in fermenting wort was not factored. Calculation of this factor has not yet been fully determined (45). Because no pressure was applied to the fermentation vessels, the dissolved amount of CO_2 resulting from temperature was therefore not enclosed. Volatile flavor compounds and panelist acceptance as well as the main flavor impressions (honey-like, blackcurrant-like, and wine-like) were chosen to evaluate the results. A quadratic model was calculated based on the experimental data consisting of linear and quadratic model terms of the factors. All the data were statistically evaluated with ANOVA. The significance of the model was evaluated with Fisher's *F* value.

Table 1. Response surface method central composite design matrix for the 17 trials performed with the two factors of pitching rate and fermentation temperature[a]

Run	Pitching rate (cells/mL)	Temperature (°C)
1*	7.5 × 10^7	21.5
2	7.5 × 10^7	26.5
3	1 × 10^8	18
4	5 × 10^7	18
5*	7.5 × 10^7	21.5
6	5 × 10^7	25
7*	7.5 × 10^7	21.5
8*	7.5 × 10^7	21.5
9	4 × 10^7	21.5
10	7.5 × 10^7	16
11*	7.5 × 10^7	21.5
12*	7.5 × 10^7	21.5
13*	7.5 × 10^7	21.5
14	1 × 10^8	25
15	1.1 × 10^8	21.5
16*	7.5 × 10^7	21.5
17*	7.5 × 10^7	21.5

[a] Asterisk (*) indicates a center point.

Response Surface for Small-Scale Fermentation

Fermentations were carried out in 2 L Duran glass bottles filled with 1.4 L of the all-malt wort diluted from extract (12.5°P, pH 5.4). Prior to pitching, the wort was aerated to 10 mg/L of DO using the same method described earlier for oxygenation demand. The inoculated samples were sealed with a GMP wireless automatic monitoring system for fermentative gas evolution (gas production monitoring) (ANKOM Technology). The pressure release of the system was set to 0 mbar until a specific density of about 4°P (approximately, loss of 50 mg of CO_2 equals decrease of 8.5°P) was reached. The decline in gravity was measured by weighing the samples every 24 h and calculating the loss of CO_2 to the specific gravity. After the samples reached 4°P, the pressure release was set to 500 mbar to ensure a CO_2 content of the final beer. After fermentation stopped the samples were stored for one week at fermentation temperature for diacetyl reduction and then cooled to 0°C for maturation for one week. They were then analyzed as described in the analytical methods section.

Analytical Methods

The alcohol content was measured using an Alcolyzer Plus with a DMA 5000 density meter and Xsample 122 sample changer (Anton-Paar GmbH, Ostfildern, Germany). Fatty acid esters were determined by gas chromatography with a flame ionization detector (GC-FID) according to the protocol shown in Table 2. Secondary metabolites were determined using headspace GC-FID analysis according to Mitteleuropäische Brautechnische Analysenkommision method 2.21.1 (Table 3). TurboMatrix 40 headspace parameters are displayed in Table 4.

Table 2. Temperature protocol and column for GC fatty acid ester determination used in this study

Device/Parameter	Setting
Column	50 m × 0.32 mm, Phenomenex FFAP, 0.25 µm
Temperature protocol	1 min at 60°C, 3 min at 220°C (5°C/min), 8 min at 240°C (20°C/min)
Detector temperature	250°C
Injector temperature	200°C

Table 3. Temperatures and column used for the GC determination of fermentation byproducts

Device/Parameter	Setting
Column	INNOWAX cross-linked polyethylene-glycol, 60 m × 0.32 mm, 0.5 µm
Oven temperature	200°C
Detector temperature	250°C
Injector temperature	150°C
Injection time	4 s
Analyzing time	17 min

Table 4. Temperatures and parameters of headspace sampling

Parameter	Setting
Sample temperature	60°C
Transfer temperature	130°C
Needle temperature	120°C
GC cycle	22 min
Thermosetting time	46 min
Pressurization time	1 min
Injection time	0.03 min

Sensory Evaluation

All beer samples were tasted and judged by a sensory panel of 10 panelists with longstanding experience in the sensory analysis of beer and certified by the Deutsche Landwirtschafts-Gesellschaft. Single tasting was performed in a dedicated tasting room (single tasting chambers, white-colored room, no distracting influences, and brown glasses) to exclude all external misleading factors. The main flavor impression intensity (wine-like, honey-like, and blackcurrant-like) was determined at a range from 1 (almost no perception) to 10 (very high perception). Flavor impressions were chosen according to prior results of fermentations with this particular strain. Here, these flavor impressions were noticed by the panelists and mentioned most in descriptive analyses (29,31). Additionally, triangle difference tests were performed to approve the differences between the produced beers.

Statistical Analysis

Results presented in this work besides RSM results are the average of three independent experiments with the bars representing the standard deviations. Data were analyzed by multiple *t* test using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, U.S.A.) to test the significance between the different mean values.

Results and Discussion

Fermentation with non-*Saccharomyces* yeast is challenging but can also lead to an innovative and newly flavored product (17,19,21). The fermentation by a yeast strain of the *T. delbrueckii* species found as a potential brewing strain was optimized by first establishing the optimal point of time to pitch from propagation. Then the oxygen demand of the strain regarding prior wort aeration was determined. Finally, the optimal fermentation temperature and pitching rate for the most desirable beer flavor were investigated by response surface central composite design.

Propagation

A high amount of pure, vital, and viable pitching yeast is required for optimum fermentation. Because yeast should be pitched when in log phase and at high vitality, the viability, cell count, and vitality were measured every 4 h after a period of 12 h of growth to predict the optimum pitching time (4,32). The viability measurement did not show great variation, as shown in Table 5.

Table 5. Viability measurement of strain T9 by propidium iodide performed throughout the propagation in triplicate with standard deviation (SD)[a]

Measurement time (h)	Viability (%)	SD
0	99	±1.2
12	97	±2.2
16	98	±0.7
20	99	±1.3
24	99	±1.1
26	99	±0.4
28	99	±1.0
32	98	±0.5
36	98	±1.5
40	98	±1.8

[a] Starting cell concentration 5×10^6 cells/mL

As shown in Figure 2, the vitality measured using the Müller-Auffermann method (32) was low, taking the yeast about 100 min to reach 690 mbar after the first 12 h of propagation. The cell number with 20 × 10^6 cells/mL (SD ±1.9 × 10^6 cells/mL) quadrupled from the start of propagation of 5 × 10^6 cells/mL (SD ±1 × 10^6). As cell numbers increased, vitality also improved up to a final level of 64 min to reach a pressure of 690 mbar. Exponential growth was detected from 20 to 36 h, reaching a cell count of 350 × 10^6 cells/mL (SD ±61 × 10^6 cells/mL). After reaching this level, cell count rose but reached the beginning of the stationary phase at around 40 h. Vitality slowly decreased after 32 h, caused by a rise in ethanol concentration of about 1% v/v and a fast decrease in nutrition (0.3°P/h) at that time (data not shown). The results show that between 28 and 32 h after start of the propagation, the time to pitch the propagated yeast for a rapid onset of the fermentation had reached its optimum, resulting from the adjusted parameters. Because cell counts of 350 × 10^6 cells/mL may seem much too high for propagated yeast it should be considered that cells are only a third of the size of *S. cerevisiae* (53). Furthermore, a rate of 100–150 × 10^6 cells/mL is chosen for *Saccharomyces* propagation because the decrease in nutrition and pH and the increase in ethanol negatively affect the yeast vitality. As shown in Figure 2, the vitality slowly decreases at about 28 h but is not significantly different from the vitality at 32 h. This gives the optimum pitching time as being between 28 and 32 h at a cell count of around 300 × 10^6 cells/mL.

Oxygen Demand

The oxygenation of wort influences yeast growth in a certain range, which is directly connected to the rate of fermentation as well as the production of secondary metabolites (10,35). To determine the optimum DO amount, five concentrations of DO (incubated with the same cell count of *T. delbrueckii* T9) between 0.2 and 20 mg/L of DO were investigated for CO_2 production, and 0.2 mg/L of DO was used as the control. The results displayed in Figure 3 show that the fermentation rate in the first 24 h significantly differed between the control (0.2 mg/L of DO) and the adjusted concentrations of oxygen of 10, 15, and 20 mg/L of DO (multiple *t* test, $P < 0.05$). Furthermore, no significant difference could be found between the CO_2 production of the sample containing 5 mg/L of DO and the control (0.2 mg/L of DO). Concentrations of 10 mg/L of DO and higher showed no significant difference in between each other (multiple *t* test, $P > 0.05$). The final attenuations of all samples (approximately 2°P, SD ±0.1) did not vary significantly, nor did the pH of the samples (approximately 4.35, SD ±0.02) (multiple *t* test, $P > 0.05$). The total CO_2 loss of all samples was determined at approximately 12.1–12.3 g. Because wort is usually aerated with sterile air, resulting in maximum DO levels of 10–12 mg/L, the results are applicable to a standard brewery (4).

Response Surface for Small-Scale Fermentation

Temperature and pitching rate were varied to predict the optimal combination for the most desirable beer aroma produced by the yeast strain *T. delbrueckii* T9. Therefore, fermentation onset, main flavor impressions (honey-like, blackcurrant-like, wine-like), secondary metabolite concentration, and acceptance were used as responses.

The fermentation lag time decreased with increased fermentation temperature as well as increased pitched cells. However, this measurement was used to predict the fermentation speed at the optimal combination of all responses. The model was found to be significant ($P < 0.05$) with an insignificant lack of fit test. Increasing the temperature from 18 to 25°C and doubling the pitching rate achieved a fermentation onset of almost three times faster (Fig. 4).

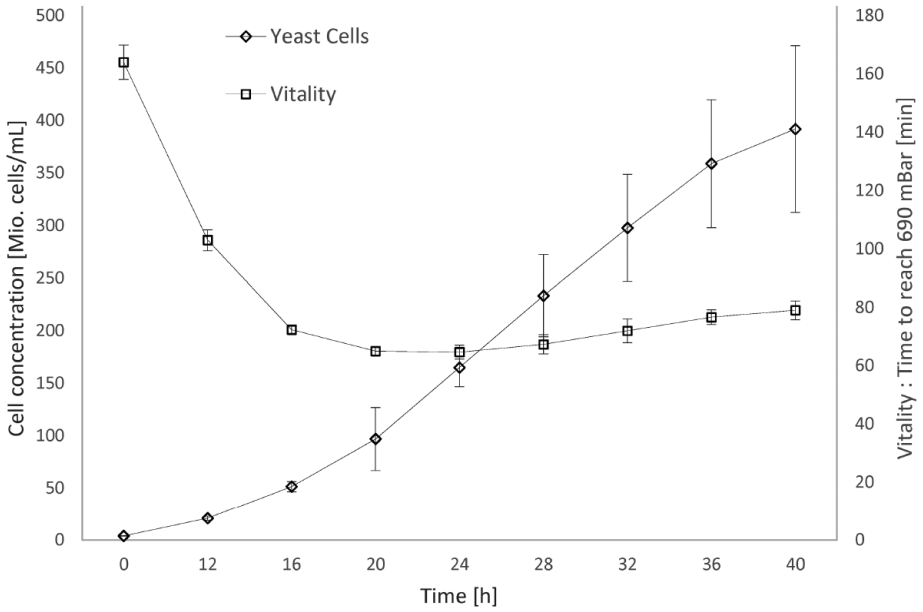

Figure 2. Measurement of vitality as a function of pressure rise to 690 mbar (minute) and cell concentration (million cells/mL) performed in triplicate over 40 h of propagation time (less time to reach 690 mbar indicates higher vitality).

The main flavor impression of the produced beers was described as honey-like at 18°C with a relatively low blackcurrant aroma. The blackcurrant aroma was highly detectable at around 21°C, changing to a strong wine-like aroma at temperatures of around 25°C. The honey-like aroma was relatively little influenced by the amount of pitched cells, as shown in Figure 5. The model was found to be significant by ANOVA *F* test statistical analyses ($P < 0.05$). The wine-like aroma was influenced most by the temperature but also a little by the cell amount, increasing slowly at increasing pitching rates (Fig. 6). The model was found to be significant ($P < 0.05$). The blackcurrant aroma was significantly influenced by the temperature, having a maximum at 21°C (Fig. 7). Here the model was also found to be significant ($P < 0.05$). All lack of fit tests were found to be insignificant, meaning that the responses fitted the model.

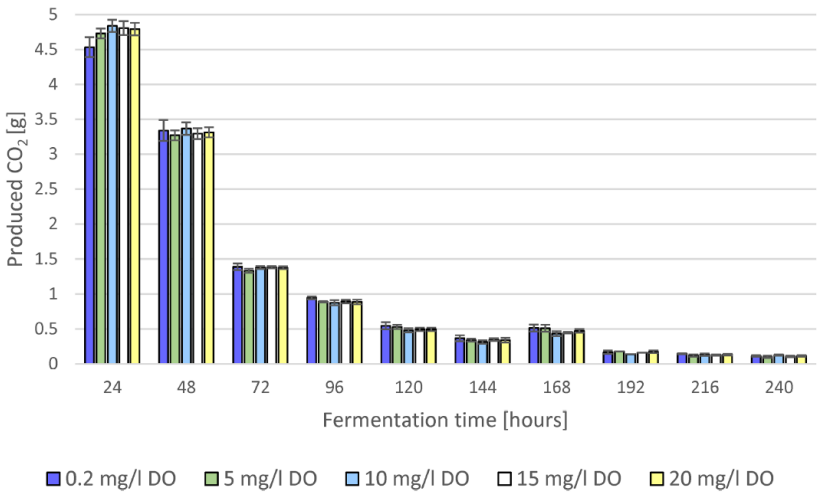

Figure 3. CO_2 production measured by weighing (loss of CO_2 described by Balling [6]) every 24 h for 10 days (240 h) with differing starting concentrations of dissolved oxygen (DO) and pitching rate at 60×10^6 cells/mL, determined in triplicate.

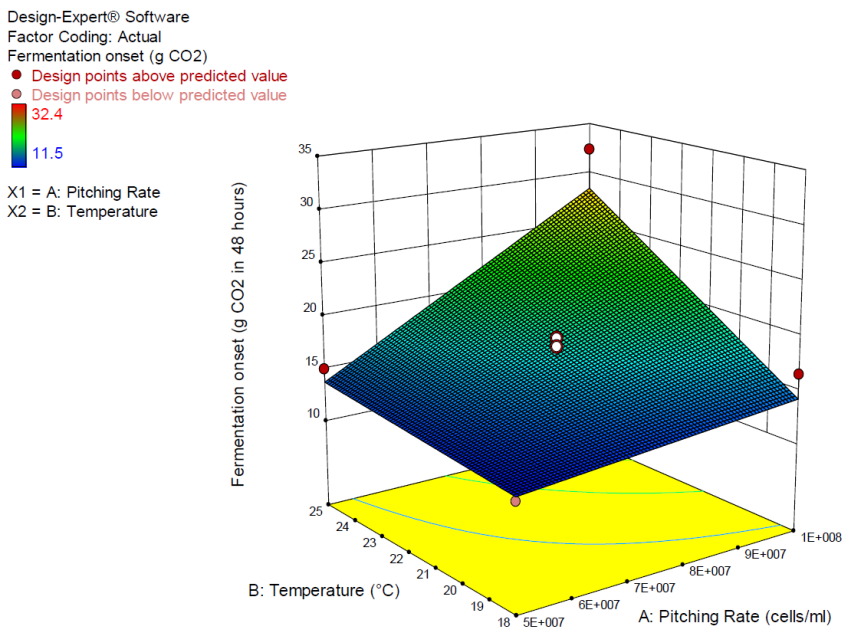

Figure 4. Response surface plot of the interactive effects of temperature and pitching rate on the onset of fermentation measured by weighing (loss of CO_2 owing to fermentation) after 48 h ($P < 0.05$).

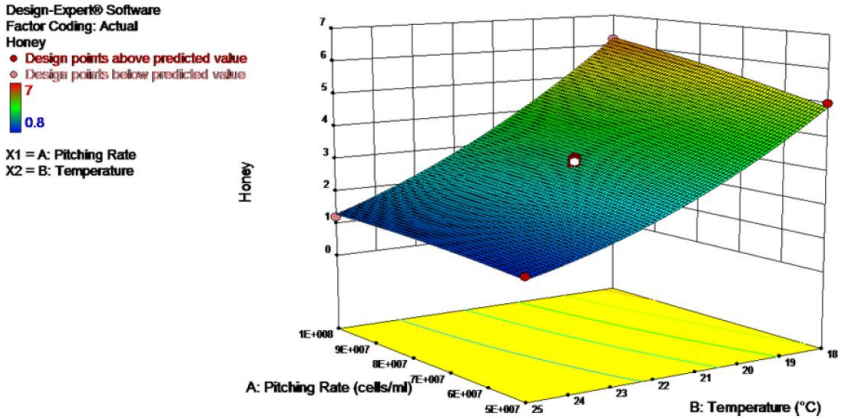

Figure 5. Response surface plot of the interactive effects of temperature and pitching rate on the main flavor impression honey-like ($P < 0.05$).

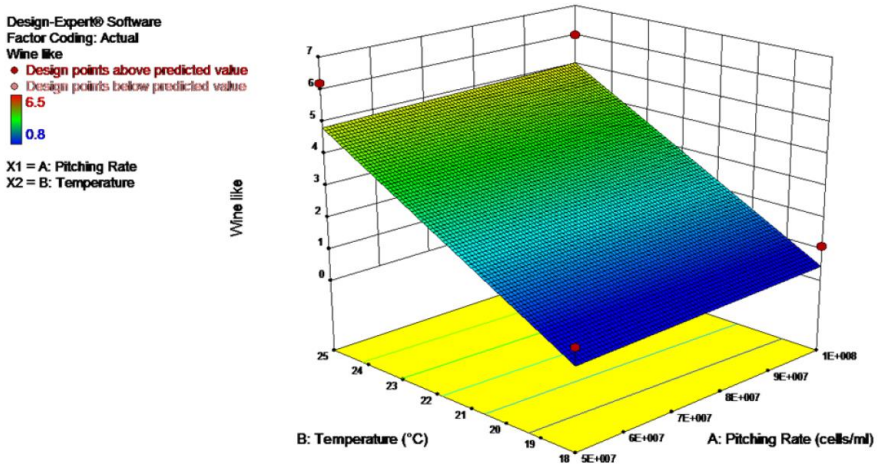

Figure 6. Response surface plot of the interactive effects of temperature and pitching rate on the main flavor impression wine-like ($P < 0.05$).

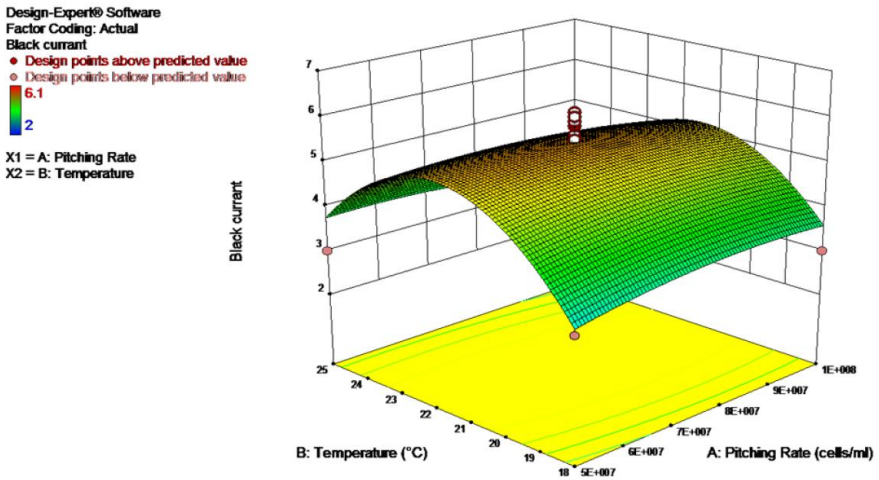

Figure 7. Response surface plot of the interactive effects of temperature and pitching rate on the main flavor impression blackcurrant ($P < 0.05$).

Total ester (butyrate, isobutyl ester, 2-phenyl ethyl ester, hexanoate, octanoate, decanoate, and ethyl acetate) formation can be seen in Figure 8. Esters are the main secondary metabolites that contribute positively to the overall beer flavor. The concentration of many of these components is in most cases relatively low. However, many authors have reported synergistic effects that these esters can take part in, resulting in the desired flavors of fruity aromas (51). The difference in impact on ester formation at changing temperatures has been reported to be owing to yeast strain as well as being species dependent (2,14). Various authors have reported an increase in acetate esters as a consequence of increasing fermentation temperature for brewing yeast (23,38,42). Some also reported a stagnation or only a slight increase by yeasts coming from other industrial fermentations (39,41). Saerens et al. showed that there were also differences between the ester concentrations themselves (42). They further reported that ethyl esters would gradually increase with increasing temperature. The yeast strain we used showed the opposite behavior, as did some non-*Saccharomyces* yeasts previously in wine fermentations (41). As shown in Figure 8, the highest amounts of total esters could be observed at temperatures around 18–19°C and a pitching rate of about 60–70 × 10^6 cells/mL. As temperatures rose, the amount of esters declined. An increase in pitching rate slightly increased ester formation.

In contrast, the total amount of higher alcohols (*n*-propanol, isobutanol, amyl alcohol [2,3-methyl butanol], and 2-phenyl ethanol) shown in Figure 9 had the highest concentration at

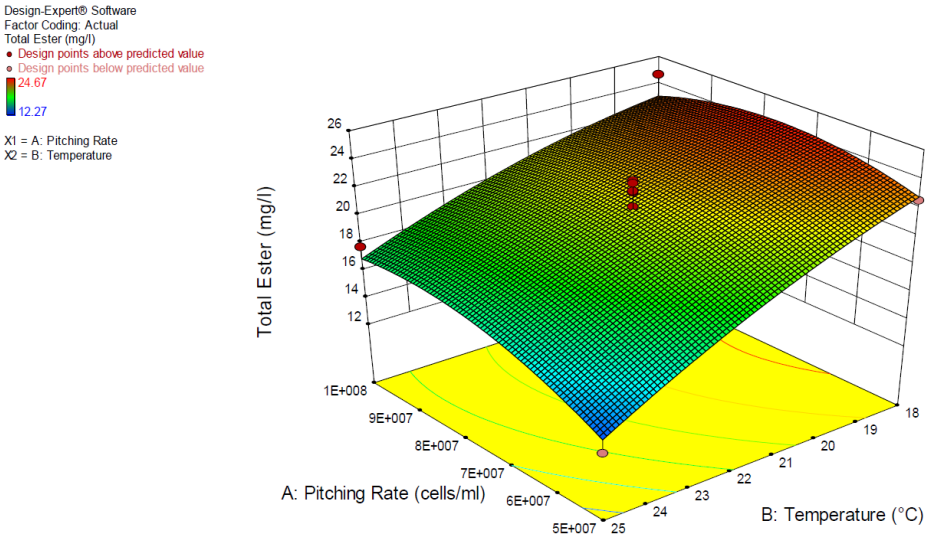

Figure 8. Response surface plot of the interactive effects of temperature and pitching rate on total ester content of the produced beers ($P < 0.05$).

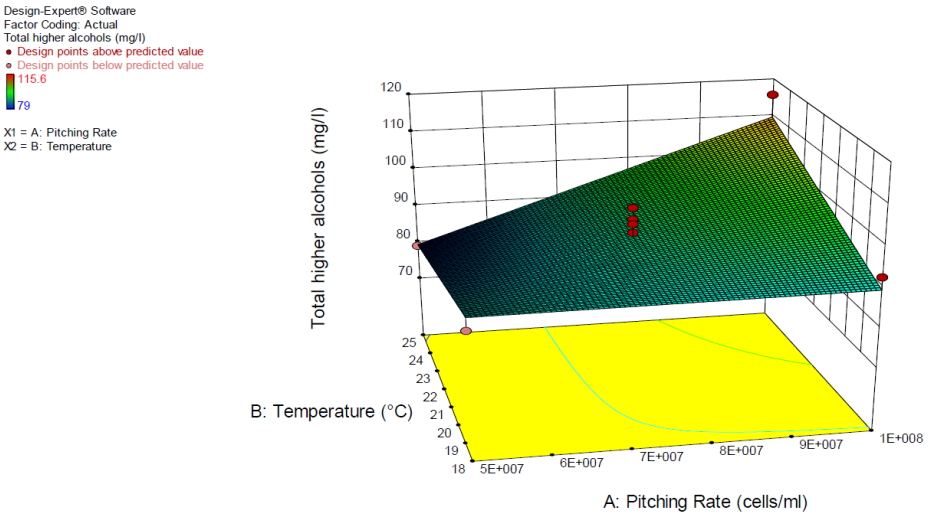

Figure 9. Response surface plot of the interactive effects of temperature and pitching rate on total higher alcohol content of the produced beers.

about 25°C and 100 × 10^6 cells/mL. It is known from the literature for brewing yeast that high fermentation temperatures can lead to high amounts of higher alcohols (37,38,51). In this case, both the temperature and pitching rate showed a significant (P = 0.018) impact on the formation of higher alcohols. Overall, the amount of higher alcohols was relatively high. Amyl alcohol concentrations reached between 60 mg/L (at 18°C, data not shown) and 70 mg/L (at 25°C, data not shown), which was well within the threshold range of 50–70 mg/L (15). The high amount of higher alcohols and the low amount of esters could explain the strong wine-like flavor that was reported for beers with high fermentation temperatures.

The statistical analysis suggested that the model was adequate. Results of the optimization of all responses showed that a fermentation temperature of about 20°C and a pitching rate of about 60 × 10^6 cells/mL would lead to the most desirable beer with the best combination for all responses. The goal was a fast fermentation with high amounts of esters and a strong honey and blackcurrant flavor impression. The optimization outcome results were verified by fermenting 50 L of the same wort, diluted from extract, in triple determination at 20°C and 60 × 10^6 cell/mL, and comparing them to the prediction of the model (Table 6). Measurements of the 50 L fermentations showed only low variation in comparison with the predicted values. The amount of medium-chain fatty acids was found to be higher than expected by the program. However, because the concentration of these compounds was low in the small-scale fermentations, no significant model could be found to fit these fatty acid values. Ester formation showed high comparability between the predicted and the measured values. Fermentation time took about 28 days to reach the final attenuation of 78%. This would conclude that the maltotriose fermentation might be low in the first pitch, as reported for many ale strains (1,52). However, the resulting beer was judged to be highly desirable by the panelists, with high values in the blackcurrant and honey main flavor impressions.

Conclusions

Varying fermentation parameters can lead to a high variability in flavors that yeast strains produce during fermentation. Fermentation outcomes can be predicted by RSM, as shown in this investigation. High desirability was found for a temperature around 21°C and a pitching rate around 60 × 10^6 cells/mL. This investigation showed further that the production and upscaling of a beer produced by a pure non-*Saccharomyces* yeast is possible and desirable. This approach can be taken with any yeast strain to screen the variability of potential flavors. Because cell sizes differ greatly between *Saccharomyces* brewing yeast and non-*Saccharomyces*, a feasible adaptation in pitching rate should be performed. For the investigated yeast strain, further studies will be conducted in the near future to upscale in larger batches and repitch to eventually improve the maltotriose fermentation. The optimization of propagation and predicting the optimal pitching time can be performed with the applied tests of vitality, viability, and cell count measurement. The focus on yeast in the brewery is rising but still has high optimization potential. Propagation has great potential for improvement. Many values such as pitching rate as well as final propagation cell counts are approximate values that vary from yeast to yeast and the performed processes.

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Table 6. Beer attributes, main aroma intensities, and ester and higher alcohol contents predicted by the model compared with the measured values in a triple determination in 50 L fermentations at 20°C and a pitching rate of 60 × 10^6 cells/mL[a]

Attributes	Response	Predicted	Measured	SD
Beer attributes	Ethanol (vol%)	5.2	5.1	±0.05
	Final attenuation (%)	79.65	78	±0.15
	pH value	4.2	4.2	±0.1
Main aroma	Blackcurrant-like	6.61	5.5	±1.21
	Wine-like	1.17	0.7	±1.54
	Honey-like	4.54	4.1	±2.10
Esters (mg/L)	Butyrate	0.37	0.32	±0.07
	Isobutyl ester	0.03	0.03	±0.01
	2-Phenyl ethyl ester	0.08	0.25	±0.09
	Hexanoate	0.03	0.05	±0.01
	Octanoate	0.03	0.02	±0.02
	Decanoate	0.03	0.01	±0.00
	Ethyl acetate	22.01	22.63	±1.76
Medium-chain fatty acids (mg/L)	Hexanoic acid	0.13	0.41	±0.06
	Octanoic acid	0.05	0.52	±0.01
	Decanoic acid	0.02	0.11	±0.05
Undesired carbonyl compounds (mg/L)	Acetaldehyde	10.41	7.00	±3.64
	Diacetyl	0.15	0.14	±0.13
Higher alcohols (mg/L)	*n*-Propanol	18.18	24.40	±2.96
	Isobutanol	12.85	14.10	±1.94
	Amyl alcohol (2,3-methyl butanol)	61.23	65.00	±2.85
	2-Phenyl ethanol	19.75	21.97	±11.48

[a] Mean, n = 3. SD = standard deviation.

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Part 4	2.5 A new approach for detecting spoilage yeast in pure bottom-fermenting and pure *Torulaspora delbrueckii* pitching yeast, propagation yeast, and finished beer

When introducing a new yeast strain into a brewery a quality control method should be used to ensure that cross contamination with other yeast strains is detected. A contamination of pitching yeast and/or of the final product can lead to undesired flavor changes, unintentional turbidity of the product or over-attenuation. Here, a method was developed to detect top-fermenting spoilage yeast in *Torulaspora delbrueckii*- and bottom-fermenting pitching yeast as well as in finished beer. A small incubation vessel with a pressure detector and a magnetic stirrer was therefore used to incubate spiked samples at 37 °C (Speedy Breedy device). Using this method, it was confirmed that *T. delbrueckii* as well as bottom-fermenting yeast will not grow/produce CO_2 at 37 °C whereas top-fermenting spoilage yeast *S. cerevisiae* var. *diastaticus* will. By providing the best growing conditions for the spoilage yeast, even very low concentration of spoilage yeast cells will grow and produce a detectable rise of pressure in the incubation vessel. The test was first evaluated by varying the pitching yeast condition, and the vitality (high and low) to investigate the possibility of false positive results through the condition of the yeast. After proving that the vitality only had a very low impact on the results, a detection level of 1.5 mbar/min pressure rise was established. Spiked samples of four different contamination rates: 10, 0.1, 0.01, 0.001 % in 1 x10^6 cells/mL pitching yeast and five different spoilage strains were used for the test. Spoilage in four different strains of bottom-fermenting yeast and four different strains of *T. delbrueckii* was investigated. All results were subsequently verified using Real-time PCR. All the spiked samples were detected as being contaminated using the new method, taking 540 min (SD±82 min) for a 10 % contamination rate and 3000 min (SD±235 min) for a 0.001 % contamination rate. No strain dependency could be found for the used pitching yeast. However, a strain-dependent detection time of the differing spoilage strains could be observed. An industrial sample was investigated to verify the method, which showed a positive result using the new method as well as in Real-time PCR.

Authors/Authorship contribution:

Michel M.: Literature search, writing, data creation, study conception and design; **Meier-Dörnberg T.**: Strain selection, support of method development; **Kleucker A.**: Support with Real-time PCR analytics and interpretation; **Jacob F.**: Supervised the project; **Hutzler M.**: Supported the creation of research plan, critical content review

A New Approach for Detecting Spoilage Yeast in Pure Bottom-Fermenting and Pure *Torulaspora delbrueckii* Pitching Yeast, Propagation Yeast, and Finished Beer

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ABSTRACT

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Beer quality is highly dependent upon yeast condition. Consequently, breweries should consider the quality of the pitching yeast. Spoilage yeasts such as wild types of *Saccharomyces cerevisiae*, *S. cerevisiae* var. *diastaticus*, and some non-*Saccharomyces* yeasts occur occasionally in pitching yeast and as secondary final-product contaminants. They negatively impact pitching yeast fermentation and beer flavor. In this study we used a novel device to measure the pressure in small-scale fermentation vessels and to detect five spoilage yeast strains of *S. cerevisiae* var. *diastaticus* in four pure pitching yeast strains of the brewing yeast *Torulaspora delbrueckii*. We investigated a method to detect the five spoilage yeasts in pure pitching yeast of four *S. pastorianus* strains. Pitching yeast was chosen for its high cell density, activity, adaptability, and therefore stress resistance, and because it could produce false positive results in the detection method. Cultivation in the vessel at 37°C in YM broth inhibited all the pitching yeasts but increased the spoilage yeast. Real-time polymerase chain reaction validated the method. Low spoilage yeast concentrations of 10 cells/mL and contaminated industrial samples were reliably detected. Minimal time was needed to prepare the sample and detect spoilage yeasts.

Keywords: Beer, Brewing yeast, Rapid detection, *Saccharomyces cerevisiae* var. *diastaticus,* Superattenuation, *Torulaspora delbrueckii*

Controlling the purity of yeast starter cultures is highly important for every brewery to ensure the quality and consistent flavor of the produced beers (10,17). Wild yeasts are one of the main contaminants (aside from lactic acid bacteria), because the strains are mainly wild *Saccharomyces cerevisiae*, *S. cerevisiae* var. *diastaticus*, and non-*Saccharomyces* yeasts such as *Brettanomyces* spp. and *Candida* spp. (3,10,21). Contaminated starter cultures can produce unwanted flavors in the final product, turbidity, and superattenuation of the final beer, resulting in lower terminal gravity, a higher alcohol content, or even exploding bottles or gushing of the product (3). Unwanted flavors are formed by a lot of *Saccharomyces* and non-*Saccharomyces* yeast including phenolic off-flavors such as solvent-like, clove-like, or Styrofoam-like flavors (8,22). In the mid-1980s, it was discovered that a concentration of spoilage yeast of 1×10^4 cells/mL had a noticeable impact on beer quality (18,22). This would lead to the beer being rejected and dissatisfied customers.

A method to detect pitching yeast contaminants in bottom-fermenting yeast has been proposed by Walsh and Martin and also by Back (2,24). The method is based on the fact that all *S. pastorianus* bottom-fermenting lager yeasts of previous investigations do not grow at 37°C. A lot of pitching yeast contaminants such as *S. cerevisiae, S. cerevisiae* var. *diastaticus*, and some non-*Saccharomyces* yeasts grow at 37°C, as shown by Walsh and Martin, by Back, and by Hutzler et al. (2,8,9,24). The methods proposed by Walsh and Martin and by Back gave reliable results in detecting spoilage yeast in the pitching yeast in 3–5 days.

The new approach described in this paper to detect spoilage yeasts in bottom-fermenting pure culture yeast uses the same mechanism. The goal of this study was to get results more easily and faster than in the original method. The original method covered cultivation of bottom-fermenting pitching or harvested yeast samples and also contaminated beer samples at 37°C on wort agar, which was preheated for 2 h at 37°C. The various colonies grown were differentiated by colony form and growth (3). In our method the pressure of a small fermentation vessel is constantly measured while yeast-containing culture medium is stirred and kept at 37°C. The small fermentation vessel is set up in the Speedy Breedy device, which measures the pressure in the vessel. Because *S. cerevisiae* and most other spoilage yeasts such as *S. cerevisiae* var. *diastaticus* are fermentative they produce CO_2, which can be measured in the headspace of the small fermentation vessel that was used in our study. A rise in pressure occurs when fermentative organisms grow inside the vessel. An increase in cell number is reflected by an increase in pressure in the vessel. The Speedy Breedy device measures the increase in pressure in mbar/min as well as the overall pressure in the vessel in millibars.

The lager yeast *S. pastorianus* TUM 34/70 is the main yeast used in beer production in Germany and is also one of the most scientifically investigated brewing yeasts (15,16). It was investigated along with the *S. pastorianus* strains TUM 34/78, TUM 44, and TUM 66.

Torulaspora delbrueckii strains were used because they are being increasingly used in breweries (increase in requests of *T. delbrueckii* from the Research Center Weihenstephan for Brewing and Food Quality, TU München [BLQ]) (8). Furthermore, Canonico et al. (2015) and Michel et al. (2016) discovered more strains for the brewing industry (6,14). No investigations have been conducted to date to detect spoilage yeast in pure culture pitching samples of this kind. *T. delbrueckii* does not grow at 37°C, as investigated by Hutzler, so identifying spoilage yeast at 37°C used this approach (8). *T. delbrueckii* strains TUM T6, TUM T9, TUM T13, and TUM T17 were chosen from the yeast center culture collection of BLQ.

S. cerevisiae var. *diastaticus* was chosen because it is one of the most common spoilage yeasts in the brewing industry that leads to superattenuation and strong off-flavors (3,9). Five strains of *S. cerevisiae* var. *diastaticus* (TUM SY 1, DSMZ 70487, TUM PIBB 105, TUM PIBA 109, and TUM BLQ 27) were chosen from the BLQ yeast center culture collection.

Four bottom-fermenting strains of *S. pastorianus*, five *Torulaspora delbrueckii* strains, and five *S. cerevisiae* var. *diastaticus* strains were investigated by using the novel method. The results were validated with real-time polymerase chain reaction (PCR).

EXPERIMENTAL

Yeast strains were provided by BLQ. Harvested yeast samples and propagation yeast samples were withdrawn from an industrial

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large-scale brewery. The investigated yeast strains can be seen in Table I. Yeast vitality was measured as acidification power (AP) according to the method of Gabriel et al. (7). Cell count was performed with a Cellometer Auto X4 cell counter (Nexcelom Bioscience, Lawrence, MA, U.S.A.). Staining with methylene blue was performed before measuring the cell count to investigate viability (4).

Medium

Yeast extract–malt extract broth (YM broth) was prepared with 3 g of malt extract, 3 g of yeast extract, 5 g of peptone, 11 g of glucose monohydrate, and 1,000 mL of distilled water. After preparation the medium was autoclaved (8).

Pressure detection was performed with the Speedy Breedy system (Bactest, Cambridge, U.K.). Disposable minifermenters (Fig. 1) and software were also supplied by Bactest. The device measures the deformation of a membrane in response to the pressure generated by CO_2 produced by microorganisms. The deformation of the membrane is directly proportional to the pressure inside the vessel. The Speedy Breedy protocol was set to rotor speed, 60 rpm; temperature, 37°C; pressure stability target, 1.5 mbar/min; and pressure stability window, 15 min. Measurements were taken for 3 days or until a significant positive signal of a spoilage organism could be detected.

Sample Preparation

YM broth (50 mL) was poured into minifermenters under sterile conditions. The medium was preheated to 37°C inside the Speedy Breedy device. Preheating was performed automatically. Inoculation was conducted after equilibration. *S. pastorianus* cell concentration and *T. delbrueckii* cell concentration were adjusted to 1×10^6 cells/mL.

The influence of yeast vitality on the response of the Speedy Breedy instrument was evaluated with samples in different physiological condition. Nine measurements were taken with three different vital stages of *S. pastorianus* TUM 34/70 measured by using the AP test to investigate if any of the vitality stages would have an impact on the growth at 37°C: high vitality, AP 2.5; good vitality, AP 2.2; and low vitality, AP 1.7 (7). *T. delbrueckii* TUM T6, TUM T9, TUM T13, and TUM T17 were adjusted to 1×10^6 cells/mL at a high vitality (AP 2.4) without spiking to investigate any growth at 37°C.

The influence of the cell count of spoilage yeast was measured by using spiked samples at different cell counts. *S. cerevisiae* var. *diastaticus* TUM SY 1 cell concentration for spiking was adjusted to 10, 100, 1,000, and 100,000 cells/mL at individual measurements performed in triplicate. Contamination rates are as follows: 10 cells/mL is equal to 0.001%, and 100,000 cells/mL is equal to 10%, compared with 1×10^6 cells/mL of pitching yeast. The defined amounts were added to 50 mL of YM broth containing 1×10^6 cells/mL of *S. pastorianus* TUM 34/70 or 1×10^6 cells/mL of *T. delbrueckii* TUM T9.

The influence of the strain of the spoilage yeast was investigated by spiking with four more strains of *S. cerevisiae* var. *diastaticus*: DSMZ 70487, TUM PIBB 105, TUM PIBA 109, and TUM BLQ 27 were spiked in concentrations of 1,000 cells/mL (ratio 1:1,000 cells) into the 50 mL of YM broth containing 1×10^6 cells/mL of TUM 34/70 or *T. delbrueckii* TUM T9 and were measured in triplicate at 37°C.

The influence of the strain of the bottom-fermenting yeast on the spoilage detection was investigated with three more *S. pastorianus* strains. *S. pastorianus* TUM 34/78, TUM 44, and TUM 66 were each inoculated at 1×10^6 cells/mL into the 50 mL of YM broth and spiked with TUM SY 1 at 1,000 cells/mL in triplicate.

The influence of the strain of *T. delbrueckii* was investigated with three more *T. delbrueckii* strains. *T. delbrueckii* TUM T6, TUM T13, and TUM T17 were each inoculated at 1×10^6 cells/mL into the 50 mL of YM broth and spiked with *S. cerevisiae* var. *diastaticus* TUM SY 1 at 1,000 cells/mL in triplicate.

TABLE I
Investigated Yeast Strains Used in This Study

Yeast	Strain
Saccharomyces pastorianus	TUM 34/70
	TUM 34/78
	TUM 44
	TUM 66
S. cerevisiae var. *diastaticus*	TUM SY 1
	DSMZ 70487
	TUM PIBB 105
	TUM PIBA 109
	TUM BLQ 27
Torulaspora delbrueckii	TUM T6
	TUM T9
	TUM T13
	TUM T17

Real-Time PCR

After every positive Speedy Breedy pressure measurement, 1 mL of sample was withdrawn from the small fermentation vessel and centrifuged for 2 min at 18,626 × *g* (Micro 200 centrifuge, Hettich, Kirchlengern, Germany). The supernatant was discarded, and the cell pellet was used in the next step. Yeast DNA was isolated with InstaGene matrix (Biorad, Munich, Germany). Real-time PCR was performed for *S. cerevisiae* var. *diasticus*, *S. cerevisiae*, *S. pastorianus,* and *T. delbrueckii* according to the methods of Brandl, Josepa et al., Scherer, and Hutzler et al. (Table II) (5,8,9,11,20). *S. cerevisiae* real-time PCR was performed to ensure the positive result of *S. cerevisiae* var. *diasticus*, because it is supposed to be identified using both systems (LightCycler 480 II, Roche, Mannheim, Germany; Taq: LightCycler 480 Probe Master [contains FastStart Taq DNA polymerase], Roche).

RESULTS AND DISCUSSION

A high vitality of brewing yeast results in a higher stress tolerance (1). To make sure the bottom-fermenting yeasts would not grow at 37°C, three measurements were taken in triplicate at different vitalities, measured by AP (7). High vitality (above AP 2.4) as described by Gabriel (7) was measured for the freshly har-

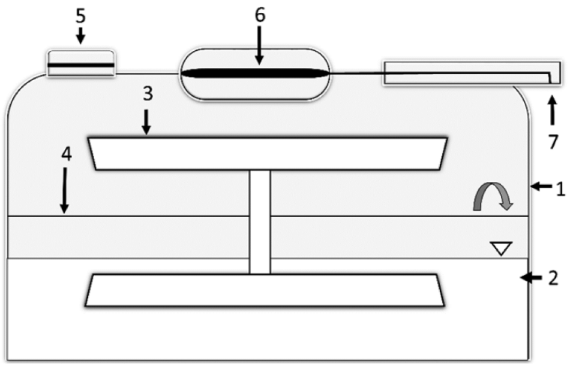

Fig. 1. Schematic diagram of the Speedy Breedy minifermenter (1): YM broth (2), magnetic stirrer (3), stirrer axis (4), inoculation rubber septum (5), measure membrane (6), and pressure-measuring connection to the Speedy Breedy device (7).

vested yeast at AP 2.5. Good vitality (between AP 2 and 2.4) was measured for the propagated yeast at AP 2.2, which had been started 24 h before with the Carlsberg flask inoculation at an industrial-scale propagation. Low vitality (between AP 1.8 and 2) was measured for the propagation yeast that was stored under beer at 20°C for 5 days at AP 1.7 (Table III) (7). None of the measurements showed a significant change in pressure in the headspace of the small fermenters after 3 days, which led to the assumption that the vitality of the yeast in that case had no negative influence on the result. The investigated *T. delbrueckii* strains TUM T9, TUM T6, TUM T13, and TUM T17 showed no growth at 37°C. This suggested no production of CO_2, and therefore a significant rise in pressure could not be observed (Table III).

S. pastorianus TUM 34/70 samples spiked with *S. cerevisiae* var. *diasticus* TUM SY 1 were measured at different cell concentrations, as seen in Table IV. The samples with a cell concentration of 100,000 cells/mL (which equals a contamination rate of 10%) were detected as being contaminated (15 min of 1.5 mbar/min pressure change) after an average of 9 h. As *S. cerevisiae* var. *diastaticus* cell concentrations decreased, the detection time increased, until a spiked cell concentration of spoilage yeasts of 10 cells/mL (which equates to a contamination rate of 0.001%) took an average of 60 h to be detected. *S. cerevisiae* var. *diasticus* DSMZ 70487, TUM BLQ 27, and TUM PIBA 109 showed similar results in detection time. TUM PIBB 105 took almost double the time to be detected but was also detected as spoilage. There was no significant difference in the detection of spoilage in *S. pastorianus* strains TUM 34/70, TUM 34/78, TUM 44, and TUM 66 (data not shown). The detection diagram of TUM SY 1 with contamination rates from 10 down to 0.001% is shown in Figure 2.

After every positive Speedy Breedy pressure detection a real-time PCR was conducted to validate the new method. Every sample measured as spoilage positive was also proven positive by real-time PCR. Figure 3 shows the three real-time PCR detections of the 10 cells/mL *S. cerevisiae* var. *diasticus* TUM SY 1. A positive control and a negative control were added to every real-time PCR run to validate the results.

The real-time PCR results showed average threshold cycle (C_t) values of approximately 25, as shown in Table V. C_t values describe the number of cycles needed to have a significantly higher reporter fluorescent signal than the average fluorescent baseline and therefore a detection of the targeted DNA. The C_t value also indicates the amount of targeted DNA in the sample. High amounts of the targeted DNA will result in low C_t values, whereas low concentrations of the targeted DNA will result in high C_t values (12). The C_t values of all the samples did not show a significant difference (Table V). This might be because a certain cell concentration has to be reached to produce 1.5 mbar/min of pressure inside the small fermenter of the Speedy Breedy device. As the C_t value is directly related to the cell concentration because it measures the concentration of target DNA in the sample, we can make a conclusion about the cell concentration. Brandl measured

TABLE II
Real-Time Polymerase Chain Reaction Systems to Identify the Investigated Yeast Strains

Target specificity	Primer	Probe/reporter/ quencher	Primer sequence (5′→3′)	Probe sequence (5′→3′)	System	Source
Saccharomyces cerevisiae var. *diastaticus*	Sd-f Sd-r	Sdia/ FAM/BHQ1	TTCCAACTGCACTAGTTCCTAGAGG GAGCTGAATGGAGTTGAAGATGG	CCTCCTCTAGCAAC ATCACTTCCTCCG	Sdi	(5)
S. cerevisiae	SCF1 SCR1	SCTM/FAM/BHQ1	GGACTCTGGACATGCAAGAT ATACCCTTCTTAACACCTGGC	CCCTTCAGAGCGTTT TCTCTAAATTGATAC	SC	(19)
S. pastorianus *S. bayanus* (partially)	BF 300E BF300M	BF/FAM/BHQ1	CTC CTT GGC TTG TCGAA GGTTGTTGCTGAAGTTGAGA	TGCTCCACATTTGAT CAGCGCCA	BF300	(20)
Torulaspora delbrueckii	Td-f Td-r	Y58/FAM/BHQ1	AGATACGTCTTGTGCGTGCTTC GCATTTCGCTGCGTTCTT	AACGGATCTCTTGGT TCTCGCATCGAT	Td	(7)

TABLE III
Yeasts Used for the Vitality Measurements with Acidification Power (AP) and Viability

Yeast	Origin	Vitality (AP)	Viability (%)	Pressure increase (mbar/min)
TUM 34/70	Harvest yeast	2.5	99	<0.5
TUM 34/70	Propagation yeast	2.2	98.5	<0.5
TUM 34/70	Propagation yeast (5 days at 20°C)	1.7	97	<0.5
TUM T6	Propagation yeast	2.4	99	<0.5
TUM T9	Propagation yeast	2.4	99	<0.5
TUM T13	Propagation yeast	2.4	98	<0.5
TUM T17	Propagation yeast	2.4	99	<0.5

TABLE IV
Average Detection Times for the Differently Spiked Samples of *Saccharomyces pastorianus* TUM 34/70

Organism	Spiked cells (cells/mL)	Contamination rate (%)	Average detection (min)	Standard deviation (min)
TUM SY 1	100,000	10	540	±82
TUM SY 1	1,000	0.1	960	±115
TUM SY 1	100	0.01	1,500	±131
TUM SY 1	10	0.001	3,000	±235
DSMZ 70487	1,000	0.1	1,020	±124
TUM PIBB 105	1,000	0.1	2,100	±134
TUM BLQ 27	1,000	0.1	1,260	±98
TUM PIBA 109	1,000	0.1	1,320	±95

C_t values and corresponding cell concentrations of *S. cerevisiae* var. *diasticus* in 2006 and reported that an average C_t value of 33 has a corresponding cell concentration of 5×10^4 cells/mL (5). Too and Anwar discovered in 2006 that a change in DNA concentration of the factor 10 will result in a C_t value change of 3.32 (23). Combining these two facts results in a cell concentration of approximately 5×10^6 cells/mL inside the fermentation test device at detection time.

The detection of spoilage in the pure cultures of the investigated *T. delbrueckii* strains gave the same results. The detection of

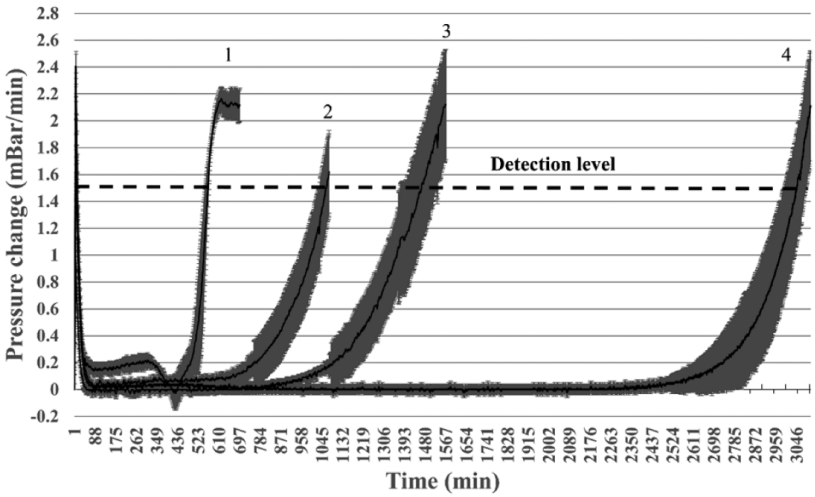

Fig. 2. Detection of four different cell concentrations of spoilage yeast *Saccharomyces cerevisiae* var. *diasticus* TUM SY 1 in pure bottom-fermenting pitching yeast *S. pastorianus* TUM 34/70 performed in triplicate with standard deviation. 1 = TUM SY 1 at 100,000 cells/mL; 2 = TUM SY 1 at 1,000 cells/mL; 3 = TUM SY 1 at 100 cells/mL; and 4 = TUM SY 1 at 10 cells/mL.

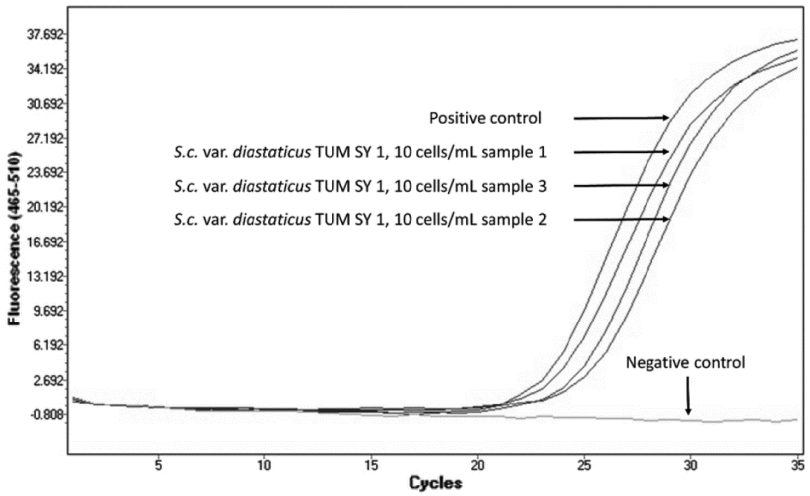

Fig. 3. Amplification curves of the three detected samples of *Saccharomyces cerevisiae* var. *diasticus* TUM SY 1 at 10 cells/mL after being detected by the Speedy Breedy device. Furthermore, one curve shows the positive control and another the negative control of the real-time polymerase chain reaction system.

TABLE V
Average Cycle Threshold (C_t) Values of the Spoilage-Detected Spiked Samples, Which Were Measured in Triplicate

Organism	Spiked cells (cells/mL)	Average C_t value at point of time of positive detection	Standard deviation
TUM SY 1	10	24.35	±0.61
TUM SY 1	100	24.68	±1.72
TUM SY 1	1,000	25.50	±0.36
TUM SY 1	100,000	25.92	±1.06

TABLE VI
Results of Spoilage Detection of the Investigated *Saccharomyces cerevisiae* var. *diastaticus* Strains in Pure Culture Yeast *Torulaspora delbrueckii* TUM T9

Organism	Spiked cells (cells/mL)	Contamination rate (%)	Average detection (min)	Standard deviation (min)
TUM SY 1	1,000	0.1	1,080	±102
DSMZ 70487	1,000	0.1	1,140	±132
TUM PIBB 105	1,000	0.1	1,980	±110
TUM BLQ 27	1,000	0.1	1,020	±93
TUM PIBA 109	1,000	0.1	1,200	±74

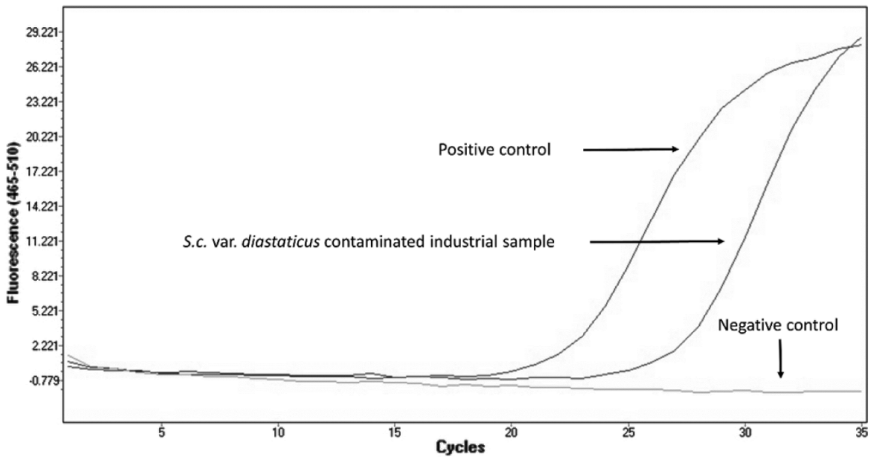

Fig. 4. Amplification curve of a contaminated industrial sample measured after it was detected by the Speedy Breedy device as spoilage. Furthermore, one curve shows the positive control and another the negative control of the real-time polymerase chain reaction system.

spoilage in a pure culture of *T. delbrueckii* TUM T9 can be viewed in Table VI.

The method used gave reliable and reproducible results. It was possible to detect spoilage of 100,000 cells/mL of *S. cerevisiae* var. *diastaticus* in 1 × 10^6 cells/mL of TUM 34/70 in an average of 9 h. The detection of 100 cells/mL was possible in 25 h.

To test the new method, it was applied to real contaminated industrial samples that were sent to our institute by a small brewery. The contaminated samples consisted of three unfiltered bottom-fermented beers stored in 0.5 L bottles still containing bottom-fermenting yeast. With the Speedy Breedy device, contamination with spoilage yeast could be identified after an average of 35 h. Real-time PCR identified *S. cerevisiae* var. *diastaticus* as the spoilage organism (Fig. 4). Comparing the identification time of spoilage with the identification times in Table IV led to the conclusion that the investigated bottles were contaminated at a rate of between 10 and 1,000 cells/mL, depending on the spoilage yeast strain. This first industrial sample trial showed good results, but more tests will have to be done to prove the method as reliable for practical use.

CONCLUSIONS

The results of the detection time show that an average time benefit can be achieved regarding the method suggested by Walsh and Martin and also by Back (2,24). It takes at least 3 days for reliable results when using agar plates. Depending on the concentration of spoilage yeast, our detection method showed results after 9–50 h. No materials were necessary other than the medium, the Speedy Breedy device, and the fermentation test device. This test can easily be performed as part of a brewery's daily routine with minimum effort. It can be applied to samples taken from all over the brewery starting with pitching yeast, the beer before and after filtering, the cropping yeast, and of course, the finished product. It is an efficient method for quality control to detect spoilage yeasts, and it works reliably under high cell concentrations of propagation yeast. Contamination rates of 0.001% of spoilage yeasts in pitching yeast and low cell concentrations in real contaminated industrial samples were reliably identified.

Further tests will be conducted with more brewing yeasts such as *Saccharomycodes ludwigii*. This strain is becoming increasingly important for the production of low-alcohol beer, but because of its maltose negativity there is a greater risk of contamination by spoilage yeast (13). Further tests with more spoilage yeasts that grow at 37°C will also be performed.

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3 Discussion

Creating, finding or adapting novel yeast strains for beer fermentation offers many opportunities along with great challenges as each yeast strain is different from each other, regardless of which genus or species they belong [7, 8, 30, 155]. Each strain has a unique ability and speed to utilize wort saccharides [127]. They behave in the opposite way when changing fermentation parameters e.g. temperature [156]. Many strains flocculate in different manners, forming thick cell agglomerates or none at all [157]. They also form a large variety of aroma compounds known as secondary metabolites [8, 9, 158]. These aroma compounds vary again by the parameters and attributes used in the fermentation [8]. Along with the unique flavor-forming ability of the strains there are synergistic effects between the different flavor compounds such as esters, higher alcohols phenols and many more [8]. Changing the yeast strain in a brewery seems to be one of the easiest steps one can take to create novel flavored beer. However, certain areas of knowledge need to be clarified in terms of the characteristics of the strain.

Most breweries have a strictly traditional way of using one or two yeast strains in their brewery. Experimentation with the yeast they use is not very common as it might change the flavor negatively or spoil other beers produced in the brewery via cross contamination [2]. The wine industry has been much more experimental, which has resulted in many desirable new aromas and innovative new wines [40, 74, 159]. As most non-*Saccharomyces* yeasts are known to brewers as spoilage yeast, the implementation of such novel brewing yeast is one of many challenges [30]. However, there is relatively little change required within a brewery when using a new yeast strain. It may just be a case of adapting the temperature of the fermentation once enough biomass has been created [105].

The main goal of this dissertation was to show how a novel brewing strain can be implemented in a brewery. Some research groups have started to investigate the use of novel non-*Saccharomyces* yeasts for beer fermentation but very little was reported on the actual use [31, 143]. Many screenings for yeast as potential flavor agents for beer or other fermented products were conducted but besides some small industrial branches there has been no real adaptation to date [2, 26, 31, 160]. As methods such as genetic modification of

microorganisms have not yet found their way into breweries, the use of natural biodiversity gives the most promising results [2].

The first part of this dissertation addressed the trials that have already been conducted on non-*Saccharomyces* yeasts in beer fermentation (section 2.2). The literature review showed that very little research was performed in the case of actual implementation of novel brewing yeasts in breweries. Most trials were limited to the fermentation of small amounts of wort by a strain with potential for use in beer fermentation [143]. Eight different species were used as pure starter cultures so far in brewing applications for alcohol-free, low-alcohol or average alcohol content beer production. They formed a variety of flavors, and showed highly different results in the fermentation speed, degree of fermentation and desirability of the finished products. Some of the species used such as *Brettanomyces anomala, B. bruxellensis* and *Torulaspora delbrueckii* presented additional benefits such as the ability to potentially change hop flavor [78, 149]. *Brettanomyces* species were able to release glyosidically bound monoterpenes from hops, increasing the amount of desired flavors coming from monoterpenes that are flavor inactive when bound to glycosides [62, 78]. It was reported that *Torulaspora delbrueckii* could transform monoterpenes, changing hop flavor as well as increasing the amount of linalool, a monoterpene responsible for a desired hop aroma, by transforming geraniol or citronellol [149] (also see section 1.5).

Across the trials, there was almost no comparability between the different studies as all of them reported different fermentation parameters e.g. pH of wort, fermentation temperature, fermentation time, pitching rate, original gravity. All of these parameters and attributes critically affect the outcome of a fermentation, making it difficult to compare them [6, 109, 161]. Table 2 presents a comparison of analyses of beers fermented by different yeast species and additionally, average German top- and bottom-fermented beers fermented with *S. cerevisiae* and *S. pastorianus* respectively. As can be seen by the table, all of the fermentations had about 3-5 % v/v ethanol, some having close to average values of secondary metabolites for top- and bottom-fermented beer. Strain *Z. rouxii* DBVPG 6463 showed above-average values for ethyl acetate, isoamyl alcohols and higher alcohols but the lowest concentration of ethanol, which was due to the condition of the used wort. They used a mashing program, which led to a high level of dextrins and less fermentable saccharides as their aim was to produce a low-alcohol beer [160]. Looking at table 2 it becomes clear that there are more brewing yeast strains than *Saccharomyces* that are capable of fermenting wort into a

respectable beer. Top-fermented beer has mostly higher than average values for most secondary metabolites due to higher fermentation temperatures [8, 161]. When comparing the conducted studies, it appeared that most of them were performed at high fermentation temperatures, giving more comparability with the average top-fermented beers. As most of the authors wanted to highlight the ability of the used strains to form secondary metabolites, higher temperatures might have been more advantageous. However, the use of novel brewing yeasts was mainly reported as having high potential for beer [31, 62, 160]. As the current number of strains, species and genera of yeast are not yet fully determined or characterized, there may still be a large number of strains with great potential [2, 30].

Table 2 Comparison of different studies of non-*Saccharomyces* fermentations of wort and average German top- and bottom-fermented beers by ethanol concentration, secondary metabolites and pH values of the final product.

	Species							
	B. bruxellensis		***B. anomala***	***Z. rouxi***	***T. delbrueckii***		***S. pastorianus***	***S. cerevisiae***
Strain code	BSI-Drie	LTQB 6	WLP 645	DBVPG 6463	T9	LTQB 7	Average beer	Average beer
Source	[162]	[45]	[162]	[160]	[142]	[45]	[15, 120, 163]	[15, 120, 163]
Original gravity [°P]	12	16	12	12	12	16	12	12
Ethanol % [v/v]	5	4	4	3	4	5	5	5
pH value	n. a.	n. m.	3.15	n. m	4.2	n. m.	4.2-4.6	4.3-4.6
Ethyl acetate [mg/L]	29.88	21.9	20.99	70.86	23.4	16.2	19	29
Isoamyl alcohol* [mg/L]	11.01	57.7	n. a.	96.2	64	69.0	38-100	50-70
Total higher alcohols [mg/L]**	26.12	97.7	n. a.	196.77	101.82	114.8	50-136	99-119
Diacetyl [mg/L]	0.03	n. m.	n. a.	0.25	>0,1	n. m.	>0,1	>0,1

*sum of 2- and 3-methyl butanol, **sum of isoamyl alcohol (3-methylbutan-1-ol), 1-propanol and isobutanol, n. m. = not mentioned, n. a. = not analyzed

Fermentation is one of the most time-consuming steps in beer production [6, 12, 19], which is why the applied characterization started with predicting the yeasts' ability to ferment wort into a respectable beer (see section 2.3). Some major requirements were therefore taken into account to identify potential brewing strains. The utilization of main fermentable wort saccharides, glucose, fructose, sucrose, maltose and maltotriose was chosen as a key

parameter, as the results give an initial indication of the level at which the yeast strain may ferment an average all barley malt wort into beer [13, 27]. Growth in the presence of hop compounds (e.g. *iso*-α-acids) and ethanol tolerance was also investigated in micro titer format. It was discovered that hop compounds, in particular *iso*-α-acids, reduce the growth of *Saccharomyces* yeast in high concentrations [107]. As no investigation has been conducted into non-*Saccharomyces* yeast to date and tolerances vary between yeast species, these tests gave substantial information on the tolerance of the used yeast strains. The tests showed that hop addition had a significant influence on the *Torulaspora delbrueckii* strains that were used. Growth was slightly inhibited by increasing *iso*-α-acid concentrations (see next page). Phenolic off-flavor tests made it possible to predict any undesired flavors coming from the decarboxylation of coumaric-, ferulic- or cinnamic acid. Furthermore, two fingerprint systems based on RAPD 21 (Random Amplified Polymorphic DNA) and RSB-PCR (Repetitive-Sequence-Based) GTG 5 were used to differentiate the strains and to prevent cross contamination. Fermentations of a standard all barley malt wort of 12 °P at the end of the screening were conducted to prove the sugar utilization as well as screen for novel flavors. As high fermentation temperatures lead to higher amounts of secondary metabolites e.g. flavor compounds [8, 161], a fermentation temperature of 27 °C was used [142].

The phenotypic characterization protocol or screening itself was found to be efficient. Nine of the ten screened strains of *Torulaspora delbrueckii* were found to be negative for maltose and maltotriose assimilation. In the subsequent fermentation of wort they showed the predicted behavior. They formed 0.8-1 % v/v of ethanol, which is a representative amount for fermenting all present glucose, fructose and sucrose from a 12.4 °P wort. The T9 strain that was found to be positive for maltose and maltotriose assimilation, fermented high amounts of the present saccharides and formed 4 % v/v ethanol. It fermented 94.8 % of a total of 55.48 g/L maltose and 58.9 % of a total of 15.41 g/L of maltotriose and 86-92 % of glucose, fructose and sucrose. These results prove the work of Alves-Araújo *et al.* in 2004 [140], who reported *Torulaspora delbrueckii* strains that had high affinity maltose transport systems that were closely related to *S. cerevisiae MAL11* as described in 1.4.2. The results further prove the high variability in saccharide assimilation in this species described by Kurtzman *et al.* [164].

However, predicting fermentation using assimilation tests before fermentation has limiting factors. Some yeast species are subject to the Kluyver effect [146]. This effect describes the ability of a strain to assimilate disaccharides aerobically but its inability to assimilate them

without the presence of oxygen. This means that these yeast strains will show a positive behavior in the first step of the characterization protocol but they will ferment poorly when pitched in all barley malt wort [165, 166]. Species such as *Kluyveromyces marxianus* are subject to the described effect. They can grow in the presence of maltose and oxygen but are unable to ferment this saccharide into ethanol once there is no oxygen. Researchers have discovered that the transport system for disaccharides like maltose (ATP requiring proton pumps described in section 1.4.2) in these yeast species do not work due to the lower availability of ATP when under anaerobic condition [12, 165, 166]. Due to the Kluyver effect some strains might therefore be unsuitable for the characterization protocol in the first place.

The addition of hop compounds resulting in 50 and 90 IBU, or 50 and 90 ppm of *iso*-α-acids did influence the growth of all ten used strains. By increasing *iso*-α-acid concentration, the growth speed decreased significantly. The total concentration of cells at the end of the growth phase, however, was not found to be significantly different. Therefore no restrictions for the fermentation of highly hopped wort can be reported. Nine strains were tolerant towards ethanol concentrations of 5 % v/v and none of the strains showed growth at 10 % v/v. The ethanol tolerance of all the used strains can therefore be described as moderate and the potential application for very high gravity brewing could therefore be excluded [12, 17]. The conducted phenolic off-flavor tests were negative for all strains as shown by the fermented beers. The flavor and aroma assessment further showed that about three of the applied strains offered desired flavor and aroma impressions. The T9 strain was described as having high fruit and floral notes, with the main flavor attribute being blackcurrant-like. The used fingerprint systems showed high uniformity along the strains. GTG 5 was more discriminative and made it possible to differentiate between the two main clusters. The RAPD 21 system showed less differentiation. However, physiological differences between T9 and all other applied strains could not be detected by the fingerprint systems.

The T9 strain that was found to be a potential brewing strain was investigated to optimize a pure fermentation of all barley malt wort by a non-*Saccharomyces* strain using response surface methodology (RSM). Prior investigations showed that the amount of pitched cells had to be higher than for the average *Saccharomyces* brewing yeasts [167]. When looking at the cell size of *T. delbrueckii* (Figure 3) on average to *Saccharomyces* brewing strains it was reported that *T. delbrueckii* cells had a mean cell diameter of 3 µm whereas the cell size of *Saccharomyces* was about 8 µm [168]. The cell size is directly related to the cell surface, which is directly related to the amount of nutrition that can be transported into the cell [102]. The

amount of nutrition that can be transported into the cell determines the amount the cell can transport, assimilate or ferment [127]. The transport and fermentation of nutrition can be regarded as fermentation performance [12]. Therefore, it seems to be a logical consequence for the amount of pitched yeast of the T9 strain to be increased in order to achieve comparable fermentation performances to the established *Saccharomyces* brewing strains.

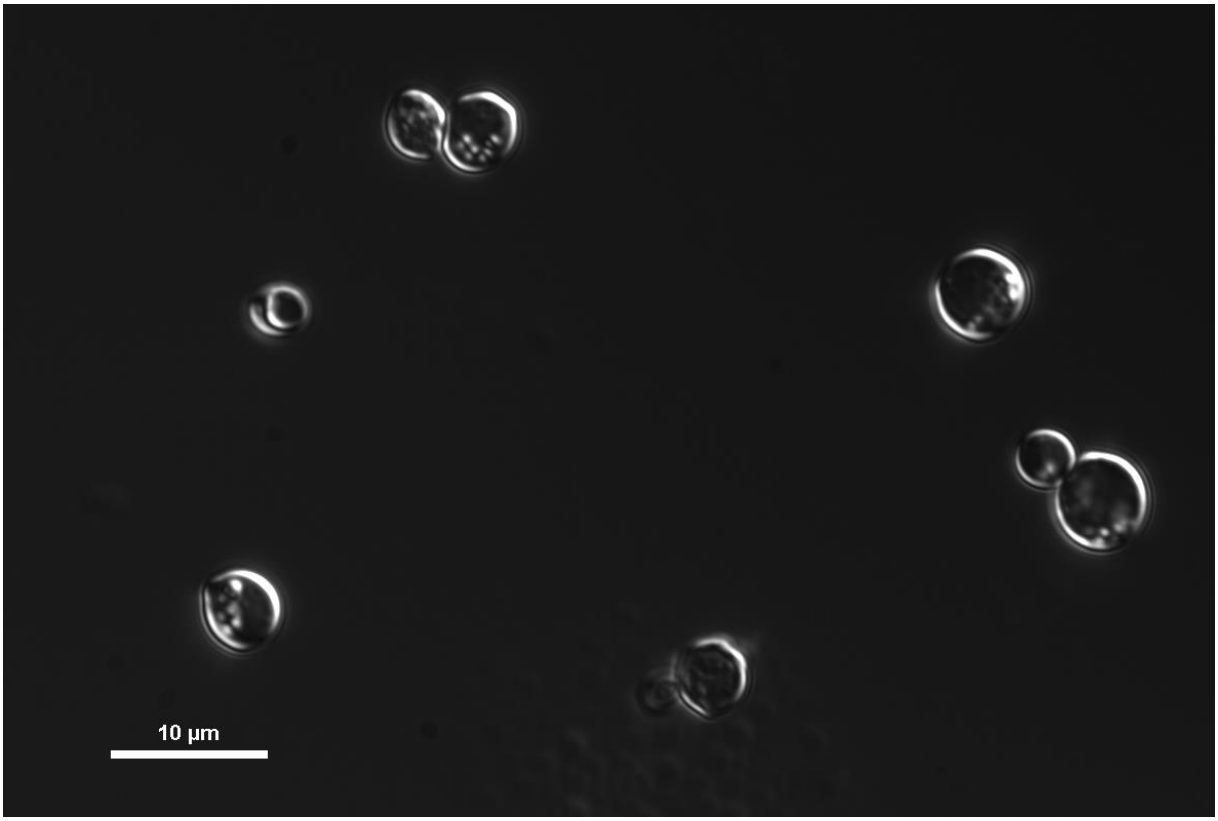

Figure 3 Microscopic oil immersion picture of *Torulaspora delbrueckii* cells, scale 10 µm, Nikon inverted research microscope Ti-E, DIC (differential interference contrast), optics: Plan Apo λ 100x Oil

Suitable propagation was essential to ferment at higher pitching rates than the usual 5-30 x 10^6 cells/mL used for *S. cerevisiae* (5-10 x 10^6 cells/mL) and *S. pastorianus* (15-30 x 10^6 cells/mL) [12, 169]. When observing the cell growth, vitality and viability of the propagation of *T. delbrueckii* it was found that concentrations of 350 x 10^6 cells/mL (standard deviation ± 61 x 10^6 cells/mL) could be achieved without a loss of vitality and viability. This made pitching between 50-100 x 10^6 cells/mL possible for the following trials (section 2.4). In contrast, it is commonly accepted that propagation of *Saccharomyces* brewing yeast should not exceed concentrations of 100-150 x 10^6 cells/mL as vitality and viability will decrease at higher cell concentrations [102, 170–172]. This is due to a fast decrease in pH value and extract which results in a rapid increase in ethanol at higher cell concentrations, producing a pitching yeast in a stressed and undesirable condition [102, 170]. A slow decrease of vitality and viability could be reported for *T. delbrueckii* after reaching 350 x 10^6 cells/mL as ethanol

concentration reached 1 % v/v, pH decreased below 4.4 and extract decreased to 9 °P. The comparably smaller cell sizes mean that higher cell concentrations at lower stress rates for the pitching yeast can be achieved. This fact is crucial when fermenting with novel yeast strains.

The wort oxygenation was hereafter investigated for the T9 strain as it is common practice to aerate the wort to a certain dissolved oxygen (DO) concentration prior to pitching [171]. The oxygen dissolved in wort is taken up by the yeast in the initial phase of fermentation as it is required to synthesize sterols and unsaturated fatty acids. These molecules are used to create new cell membranes, as yeast at that time is multiplying. The cells can multiply until the sterol level or another growth factor limits growth [12]. There are, of course, more factors that contribute to the growth of yeast such as nutrition concentration or the concentration of inhibitory substances such as ethanol, because of which a certain maximum aeration level will not significantly increase growth. After reaching this maximum amount, no effect can be observed for the growth of the yeast population. There are reports by different authors that oxygen is required in different concentrations for different yeast strains [170, 173]. The main difference in wort oxygenation is between top and bottom-fermenting yeast. For bottom-fermenting yeast a DO level of 8-9 mg/L is suggested, for top fermenting yeast it is 4.5-6 mg/L DO [174]. A level of 4 mg/L DO has been described as low, resulting in less growth and therefore a decrease in fermentation performance [102]. The oxygenation of wort also has an effect on ester synthesis [175, 176]. An inverse correlation between cell growth and ester synthesis has been reported. Whereas the described cell growth increases with increasing oxygenation [102]. This interaction may be part of the reason why many different authors report different oxygenation rates for wort because a balance in cell growth and ester production has to be determined [173, 174]. The method used in this dissertation (section 2.5) aimed to maximize fermentation performance as a result of cell growth. Five concentrations of DO of 0.2, 5, 10, 15 and 20 mg/L were investigated with 0.2 mg/L DO as a control for no oxygenation at all. The *T. delbrueckii* T9 strain showed significant differences in growth in the first 24 hours in between the control and the 10 mg/L DO samples ($p<0.05$). The rate of fermentation measured by the production of CO_2 showed maximum values for the wort samples oxygenated to 10 mg/L DO. After 24 hours, no significant difference could be observed between the fermentation performance of the T9 yeast strain in the five different concentrations. It was concluded that 10 mg/L DO represented sufficient wort oxygenation for a fast onset of fermentation as a result of maximum cell growth.

In the following investigation, pitching rate and fermentation temperature were varied using RSM Central Composition Design (CCD), as these two factors are known from literature to have a great impact on the fermentation performance and flavor forming of brewing yeast strains along with oxygenation [20, 102, 156, 161, 177]. The results show that it was possible

to predict the fermentation outcomes using the response surface methodology in the case of taste and secondary metabolites. The fermentation with the investigated T9 strain also showed desirable flavor as well as average fermentation performance. The results further showed that a change in fermentation temperature had a greater impact on the flavor of the produced beers than the pitching rate. A fermentation temperature of 15 °C led to a honey-like taste as confirmed by the tasting panel. These flavors might come from esters such as 2-phenylethyl acetate, which has been described as having a honey-like flavor [178]. Although the concentration of this particular ester was not above the threshold, the total ester content was relatively high. A synergistic effect of many esters might lead to an aroma impression like that [8, 9]. In contrast to reports about *Saccharomyces* brewing yeast, the total ester content was found to be higher when fermenting at lower temperatures (15 °C). This effect has been previously reported for wine yeast strains coming from genera other than *Saccharomyces* [179]. The main flavor of beers changed at increasing temperatures towards blackcurrant-like at fermentation temperatures of 20-21 °C. As fermentation temperature was increased to 25 °C, a strong wine-like flavor of the beer was reported by the panelists. When comparing ester and higher alcohol content it became apparent that the ratio was almost the opposite to the 15 °C sample. The total ester concentration decreased from about 24 mg/L at 15 °C to 12 mg/L at 25 °C at a constant pitching rate of 60 x 10^6 cells/mL. It should be noted that ethyl acetate mainly contributed to the quantity of total esters by about 90 %. However, as many synergistic effects are not yet understood and many flavor-active substances that contribute to beer flavor have yet not been determined, it is not possible to conduct a full evaluation [9, 10]. The overall highest acceptance by the panelists was found to be a combination of 20-21 °C fermentation temperature and a pitching rate of 60 x 10^6 cells/mL. When performing three 50 L fermentations with these parameters these results could be verified. The predicted results closely matched the results of the fermentations.

Lastly, a detection method for *S. cerevisiae* var. *diastaticus* (representative for *S. cerevisiae* brewing and wild yeast positive for growth at 37 °C) in *T. delbrueckii* and *S. pastorianus* pitching yeast was introduced (section 2.5). As cross contamination from other yeast strains can significantly change the aroma profile, turbidity and attenuation of a beer [180], early detection helps prevent off-flavors in beer and upset customers [181, 182]. As already mentioned, most brewers are not keen on implementing novel brewing strains due to fear of cross contamination. This method was an approach to lower the barrier of implementing a novel brewing strain. For the applied method, a device was used that was able to stir, incubate at constant temperature, and measure the pressure of the fermentation vessel (Speedy Breedy, Bactest®, Cambridge, UK). The combination of these three attributes made it possible to incubate at 37 °C, constantly mix the sample and the cultivation media for fast growth, and detect growth of spoilage yeast in one to five days via CO_2 production. In contrast to standard

methods such as agar plate and multiple inoculation steps, the method used gave faster results with less effort and could be used in the brewery.

The underlying theory was that *T. delbrueckii* and *S. pastorianus* do not metabolize at 37 °C unlike *S. cerevisiae* [183, 184]. Even highly vital and viable pitching yeast of *T. delbrueckii* and *S. pastorianus* was unable to adapt to this temperature as proven by the conducted trials. As propagation is meant to provide optimal growth condition for yeast, spoilage yeast can of course latently grow, as their cell concentration is relatively low in the beginning. However, it is possible that their impact will increase in the fermentation as well as in the finished beer. *S. cerevisiae* var. *diastaticus,* for example, can form POF (phenolic off-flavors) as well ferment dextrins as mentioned in section 1.4.2. The spoilage result can have a negative impact on the flavor as well as over attenuation of the beer, resulting in over carbonation and in the worst case scenario, exploding bottles [135, 136]. As novel non-*Saccharomyces* brewing yeast might have some disadvantages towards fermentation against highly adapted spoilage or even conventional brewing yeast, cross contamination should be avoided from the start. The applied method can be used to detect all kinds of spoilage and wild yeast that grow at 37 °C.

In summary, the introduction of a novel brewing strain has been successful. As the amount of uncharacterized strains cannot yet be overlooked, there is no prediction of how many novel yeast strains can be implemented. As their use in the brewery can be in different fields and the aroma profile of almost all strains vary greatly, there is a great potential for these novel strains to increase the overall aroma of beer again.

4 References

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5 Appendix

5.1 Non-reviewed papers

- Hutzler M., Stretz D., Meier-Dörnberg T., Michel M., Schneiderbanger H.: “Großes Aromapotenzial – Die vielfältige Aromenwelt der Hefen”. Brauindustrie 4: 28 - 31, 2015
- Michel M., Zarnkow M., Jacob F., Hutzler M. : “Die Hefe - Kleine Lebewesen- große Helfer der Brauer” Sonderausgabe Brauindustrie 500 Jahre Reinheitsgebot: 26-29, 2016
- Michel M., Zarnkow M., Jacob F., Hutzler M. : “Die Hefe- Kleine Lebewesen- große Helfer der Brauer” Getränkefachgroßhandel 500 Jahre Reinheitsgebot: 26-29, 2016
- Meier-Dörnberg T., Schneiderbanger H., Michel M., Hutzler M., Jacob F.: Hefe als neuer Geschmacksmotor der Brauindustrie – Charakterisierung unterschiedlicher Hefestämme für den industriellen Einsatz- Der Weihenstephaner 84, S. 22-25, 2016
- Michel M., Meier-Dörnberg T., Jacob F., Hutzler M. : “Die Suche nach neuen Brauhefen abseits der *Saccharomyces”* Brauwelt 156: No.19-20, S. 555-558 2016

5.2 Oral presentations with first authorship

- Michel M., Hutzler M., Jacob F.: “Potential und Charakterisierung von Nicht-*Saccharomyces* Brauhefen für die Brauindustrie” 3. Seminar Hefe und Mikrobiologie, Freising Weihenstephan, 2015
- Michel M., Hutzler M., Jacob F.: “ Developing a screening system for the brewing ability of non-*Saccharomyces* yeasts” MBAA Annual Conference Jacksonville Florida USA, 2015
- Michel M., Hutzler M., Jacob F.: “Non-*Saccharomyces* yeasts in brewing” 26. Pivovarsko Sladařské DNY, Olumuc Czech Republic, 2015
- Michel M., Jacob F., Hutzler M.: “Nicht-*Saccharomyces* Bierhefen - Eine neue Option“ 4. Seminar Hefe und Mikrobiologie, Freising Weihenstephan, 2016
- Michel M., Jacob F., Hutzler M.: “Screening for the brewing ability of non-*Saccharomyces* yeasts” Young Scientist Symposium, Chico California USA, 2016
- Michel M.: “Nicht-*Saccharomyces* Hefen und zur Bierherstellung – praxisrelevante Ansätze“ 5. Seminar Hefe und Mikrobiologie, Freising Weihenstephan, 2017

5.3 Poster presentations with first authorship

- Michel M., Koob J.: “Heat resistance and adaptation of three beer spoilage bacteria” Young Scientist Symposium, Ghent Belgium, 2014
- Michel M., Jacob F., Hutzler M.: “Developing a screening system for the brewing ability of non-Saccharomyces yeasts” Trends in Brewing, Ghent Belgium, 2016
- Michel M., Meier-Dörnberg T., Zarnkow M., Jacob F. Hutzler M.: “Screening for the brewing ability of non-*Saccharomyces* yeasts and optimization of fermentation performance of one *Torulaspora delbrueckii* strain found suitable for beer production” WBC, Denver USA, 2016
- Michel M., Meier-Dörnberg T., Wagner R. S., Jacob F., Hutzler M.: „Novel spoilage yeast detection method for bottom fermented beer and pitching yeast“ 36th EBC, Ljubiliana Slovenia, 2017

5.4 Permission of publishers for imprints of publications

Michel M., Meier Dörnberg T., Jacob F., Methner F., Wagner R., Hutzler M. (2016). "**Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications.**" Journal of the Institute of Brewing & Distilling 122: 569-587 DOI: 10.1002/jib.381

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